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(54) Title: IMMUNOASSAY FOR HORMONE DETECTION

(57) Abstract

The invention relates to the field of endocrinology, more in specific to the field of (steroid) hormones and to (immuno)assays capable of detecting (steroid) hormones in samples and also to the field of immunoassays. The invention provides a method to eliminate the interference of hormone binding protein with a hormone for use in an immunoassay which is capable of measuring for example a hormone level in a biological sample which sample contains a specific plasma binding protein reactive with a hormone.

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Title: Immunoassay for hormone detection.

The invention relates to the field of endocrinology, more in specific to the field of hormones and to (immuno)assays capable of detecting hormones in samples and also to the field of immunoassays. Typical examples of such hormones are T3 and T4, prostaglandines, steroid hormones such as testosterone, aldosterone, progesterone, dexamethasone, corticosterone and cortisol, various peptide hormones such as glucagon, insulin, gastrin, secretin, ACTH, LH, FSH, TSH, TRH, LHRH, vasopressin, IGF-I or II, EGF, somatotropin (growth hormone), prolactin, erytropoietin, EGF, cytokines, and others.

For example, steroid hormones and small protein hormones play an important role in regulating and maintaining a wide array of bodily functions in animals and man. Although each specific hormone can often be assigned a special function, they often have overlapping action. An interplay of steroid hormones is essential in, among others, regulating reproductive processes such as gametogenesis and ovulation, immune reactions, inflammatory responses, diuresis, and regulation of stress. IGF-I and II, like insulin, has metabolic effects and stimulates (skeletal) growth. IGF-I or II are often expressed at high levels in various tissues and organs such as lung, kidney, testis, ovary, CNS. Adequate follicle development is among others mediated via IGF-I levels in the blood. Through the granulosa cells it influences the production of oestradiol, inhibitin and other growth factors. Insulin-like growth factor I and II (IGF-I and IGF-II), play an important role in growth and differentiation. The polypeptides are highly conserved as for example shown by the identical amino acid sequences of human, bovine and porcine IGF-I; in ovine IGF-I one amino acid is substituted (Etherton, 1991). Six specific high-affinity IGF binding proteins (IGFBPs) are present as binary and ternary complexes of approximately 30-50 and 150 kDa in the

circulation and other body fluids and frequently in high concentrations in cell culture media (Hossenlopp et al., 1986; Baxter, 1991; Shimasaki et al., 1991a,b; Jones & Clemmons, 1995). IGFBPs interfere in immunoassays for IGF and therefore their complete removal or inactivation after separation from IGF is necessary. So far several methods were used to separate IGF from its binding proteins before analysis including acid size exclusion chromatography (ASEC), acid-ethanol and ethanol-acetone-acetic acid extraction, sometimes combined with cryo-precipitation, glycyl-glycine extraction and Sep-Pak chromatography. ASEC is in general considered to be the optimal procedure, however, none of these methods completely removed or inactivated the IGFBPs (Humbel, 1990; Blum & Breier, 1994; Frey et al., 1994; Frystyk et al., 1995).

Measuring individual hormone levels in biological samples obtained from captured or domesticated animals or man can give significant information on for example reproductive functioning (progesterone, estrogens, testosterone), stress (cortisol, aldosterone), or production parameters such as growth, body condition, energy balance and milk yield(IGF-I or II, growth hormone, insulin, prolactin) at the time of sampling. Knowing such levels is for example helpful in animal husbandry, wild-life- or zoo-management and human medicine when hormone related conditions, such as reproductive failure, stress, immune responses, need to assessed. Also, in experimental and/or small animals, such as primates, mice, rats, dogs, cats, rabbits and birds, assays that can measure hormone levels, would be of use. However, rapid and easily applicable assays that can accurately detect or measure the presence and/or level of a specific hormone of a wide variety of species which is circulating in the blood at the time of sampling are in general not available.

Circulating hormones such as steroid hormones or small protein hormones are, in general, bound to specific binding plasma proteins (BPP) or binding globulin (BG), having

specific reactivity and often species specificity for each hormone. The fraction of free circulating hormone is, in general, small, and inaccurately reflects the total bound fraction which is circulating. Such hormones can also be detected in other samples than blood, such as saliva, milk or urine, wherein (most of) the hormone is not bound to a BPP; hence many (immuno)assays have been specifically designed for such, non-blood derived, biological samples. However, such assays do not function satisfactorily when testing plasma, serum or whole blood samples, or samples contaminated with serum or plasma or substantially containing BPP.

Hormone measurements in biological samples have traditionally been made using radio-actively labelled hormone derivatives or radio-actively labelled antibodies as tracers. However, the environmental and health consequences of such radio(immuno)assays have made the use of non-radio-active assays desirable, hence other assays, in general immunoassays employing antibodies (for example labelled or detected with reporter molecules such as biotin or horseradish peroxidase) specifically reactive with a (steroid) hormone, have been developed. However, two problems related to the sensitivity and the specificity of such immunoassays need to be overcome to be able to apply immunoassays widely to the detection of (circulating) hormones in a wide variety of samples originating from a wide variety of species.

The sensitivity and specificity of immunoassays is greatly hampered by the fact that most hormone is bound to its (species) specific BPP, thereby escaping detection by the specific antibody. Examples of such BPP are testosteron binding globulin (TeBG), also called sex hormone binding globulin (SHBG OR CBG). Another example, that also binds steroid hormones is corticosteroid binding globulin (CBG). T3 and T4 are bound by TBG, small peptide hormones can bind to a variety of BPPs. Use of classical hormone releasing agents, intended to liberate the hormone from its BPP, which have been traditionally employed, does not have a general effect

on all species specific BPPs and can even be counterproductive in an immunoassay. Immunoassays using non-blood derived samples, such as saliva or urine, circumvent this problem, but have the disadvantage that the fraction of hormone present in the sample inaccurately reflects the level of circulating hormone, thereby only approximating true values. Furthermore, contamination of such a sample with plasma, serum or whole blood (which can often be the case with these fluids) also contaminates the sample with BPP, thereby rendering the assay even more inaccurate and less sensitive than needed.

In order to cope with the (unwanted) low sensitivity and low specificity of such immunoassays, often one has used one of the most sensitive non-radioactive assay systems available, the so-called time-resolved fluoroimmunoassay. Such assays, which are not only used for the detection of steroid hormones but have much broader application, often use a streptavidin-Europium complex as a generic reagent to detect biotin-labelled compounds, e.g. antibodies or antigens in various types of immunoassays. However, although it has been reported that streptavidin shows less non-specific binding (NSB) than for example avidin, we detected, when using streptavidin-Europium under established immunoassay conditions, i.e. using bovine serum albumine (BSA) containing compounds as a NSB blocker, sufficient NSB to significantly hamper the assay. Thus the need exists to further reduce the NSB in such away.

The invention provides a method for reducing the binding of a hormone binding protein (such as BPP or BG) with a hormone presenting a sample which method comprises heating of said sample. A method for determining the presence of a hormone in a sample is provided by the invention, wherein said sample is possibly containing a binding protein for said hormone, said method comprising reducing the binding between said hormone binding protein and said hormone by heating said sample and further comprising detecting the presence of the

hormone not-bound to said binding protein. The invention provides a method wherein the binding protein is a binding plasma protein such as a sex hormone binding globulin (SHBG OR CBG) or an insulin-like growth factor binding protein (IGFBP). A preferred embodiment of the invention is a method wherein said sample is pre-heated. The invention provides a method wherein, for the purpose of detecting the hormone, the binding protein is denatured more rapidly than the hormone, thereby allowing for a test reagent capable of detecting the hormone, such as a specific antibody, to not be hindered in its detection by the interference of the binding protein. By selecting an appropriate temperature and time during which the sample is heated, the large binding protein denatures more rapidly than the small hormone, thereby leaving the hormone to be detected by for example an antibody. Said denaturing should, for the purpose of an immunoassay be rather mild, since it is unwanted that the antibodies used be denatured as well, thus abolishing the assay totally.

The invention herewith provides a method allowing a more accurate and sensitive determination of a hormone present in a test sample, without having to take into account the effect of the hormone binding protein. One method according to the invention is provided for reducing the binding of a steroid hormone binding protein with a steroid hormone. In the experimental part of this description, a method provided by the invention is explained further in detail, wherein the hormone is cortisol. A method according to the invention is provided for reducing the binding of a hormone binding protein with a small peptide hormone. In the experimental part of this description, a method provided by the invention is explained further in detail, wherein the hormone is insuline-like growth factor (be it IGF-I or IGF-II).

The invention provides a method which comprises heating a biological sample such as saliva or urine, hereby allowing for the accurate determination of hormone in for example saliva or urine samples that have substantially been

contaminated with a hormone binding protein because the samples contain plasma or serum, for example as a result of the sampling method. Especially such samples from animals, that can not be informed about the sampling, and from which samples often have to be taken while constraining the animal, are often substantially contaminated with a hormone binding protein due to inadvertent bleeding during sampling. A preferred embodiment of a method provided by the invention is wherein a sample consists of serum or plasma, samples wherein the presence of a hormone binding protein otherwise always lead to the inaccurate determination as hormone levels. Yet another embodiment of the invention comprises a method according to the invention wherein the sample is a cell-culture sample. In this way, the hormone levels in cultures of (heterologous) cells or expression systems expressing a hormone can be tested without interference with a BPP present in the culture. A method provided by the invention can additionally comprise cooling said sample to ambient temperature after heating, thereby allowing for a convenient handling time of the samples, so that determination of hormone levels can be performed with no rush. Also, the invention provides a method which additionally comprises adding to said sample a denaturing or blocking agent, such as sodium salicylate or trichoroacetic acid. In this way, the invention provides a method wherein a small peptide hormone is additionally protected against denaturation due to heating at higher temperatures by the addition of a said denaturing agent. Said additional denaturing should, for the purpose of an immunoassay be rather mild as well, since again it is unwanted that the antibodies used be denatured as well, thus abolishing the assay totally. Strong denaturing agents as urea and dithiothreiotol are under most circumstances thus not suited when not diluted properly.

Further to the problem of serum binding protein interference which is encountered in many steroid immunoassays, with small peptide hormones an additional problem exists. The

peptide hormone as well as the binding protein are sensitive to denaturation by heating at the relatively low temperatures provided by the invention. In contrast, steroid hormones are relatively insensitive to said heat treatment. The invention now provides that due to the differences in size and structural complexity of the small peptide hormone (such as IGF-I), as compared to the respective BPP, the former is rendered less temperature sensitive than the latter. Furthermore, the invention provides a method which additionally comprises diluting said sample with water, thereby allowing for the determination of hormone levels at various dilutions of a sample, which further can enhance the sensitivity, and accuracy. A preferred method according to the invention is a method wherein said sample is derived from a captured or domesticated animal as explained above, samples of animals are more likely to be contaminated by a hormone binding protein, and more importantly, the invention provides a method wherein the species specifically other hormone binding protein is not of influence any longer. Also, the invention provides a method to reduce non-specific background staining found with an immunoassay using (strept)avidin which method comprises reducing the use of or not using BSA and/or comprises the use of heparin in said immunoassay. By reducing BSA, or not using it, the non-specific binding of (strept)avidin is greatly reduced, thereby further enhancing the specificity of the assay. A preferred method according to the invention is a method wherein the immunoassay is a (time-resolved) fluoroimmunoassay using (strept)avidin labelled Europium, thereby further enhancing the sensitivity of this non-radio active test system. Furthermore, the invention provides use of a method according to the invention in an assay to detect the presence of and/or determine the level of a hormone in a sample. A preferred use is a use wherein the hormone is a steroid hormone such as cortisol or a small peptide hormone such as IGF-I, as further explained in the experimental part of the description. Furthermore, the

invention provides an assay which is capable of measuring a (steroid) hormone level in a biological sample which sample substantially contains a specific binding plasma protein reactive with said hormone. A preferred assay according to the invention is an immunoassay, preferably an enzyme-linked immunoassay or a (time-resolved) fluoroimmunoassay. A preferred assay according to the invention comprises a use of a method according to the invention to eliminate the interference of species-specific hormone BPP, present in the sample in plasma, serum or whole blood, with the hormone to be determined. Contrary to what has been taught before, pre-treating steroid hormone and BPP containing fractions with steroid hormone releasing agents does not or only partly liberate the hormone from the binding protein, while BPPs of different species show different activities as well. In general these differences reduce the sensitivity of the subsequent assay for samples of various origin. The invention provides an immunoassay or method that is applicable with human serum or plasma samples as well as with serum or plasma samples obtained from captured and/or domesticated animals. A method provided by the invention comprises pre-treating the serum or plasma samples by heating, for example in a water-bath at a predetermined temperature for a certain time. The exact times and temperatures, which are species dependent, can easily be determined for each and every embodiment of a method provided by the invention. In cortisol testing, preheating human serum or plasma samples for at least 15 minutes at at least 60°C or equivalent conditions, allows elimination of the interference of the cortisol BPP, whereas for bovine serum or plasma samples preheating for at least 15 minutes at at least 70°C, more preferably at least 30 minutes at at least 80°C, allows said sufficient elimination to reliably and accurately test the sample for the presence of the hormone. For serum or plasma samples originating from other animals, such as pigs, chickens or rabbits, or for other hormones or binding proteins, yet other specific

preheating conditions can be found by assaying these as explained in the experimental part. An additional method provided by the invention comprises diluting the test samples in water. It was found that when samples were diluted in buffer (which is standard practice in immunoassays) preheating up to certain temperatures resulted in solidification of the samples, thereby making it impossible to further test the samples, diluting the samples in water solves this problem. Furthermore, the invention provides a method to reduce the non-specific background staining (NSB) found with (time-resolved) fluoroimmunoassays using streptavidin-Europium (EUlabelled Streptavidin). Reducing NSB by a method provided by the invention is surprisingly simple but goes against the grain of conventional wisdom and practice in the field of immunoassays that often use BSA in differing concentrations in coating fluids, buffers and/or washing fluids employed to quench NSB. A method provided by the invention comprises reducing or even abolishing use of BSA as NSB blocker in fluoroimmuno-assays using streptavidin-Europium. Alternatively, to the assay buffers heparin is added when BSA cannot be omitted, surprisingly it was found that heparin counteracted the effect of BSA.

The invention further provides an immunoassay which is capable of measuring a hormone level in a biological sample which sample substantially contains a specific plasma binding protein reactive with said hormone. Such an immunoassay provided by the invention is preferably used to measure the hormone level in plasma, serum or whole blood samples, but can also be advantageously used to measure said level in samples, such as saliva or urine, that may be contaminated with plasma, serum or whole blood, or in cell culture samples. The invention for example provides use of a method according to the invention for testing human samples for IGF-I levels that are associated with risks on development of prostate cancers or other cancers as recently described by Chan et al (Science, 279:563-566, 1998). An example of the

invention is given in the experimental part of the description which describes an immunoassay capable of measuring steroid hormone levels in bovine samples, of which it was for example found that bovine BPP showed a stronger interaction with cortisol than human BPP. An immunoassay according to the invention is capable of accurately testing a variety of steroid hormones, in the experimental part, the invention is further explained by using cortisol and IGF detection as an example.

The invention also provides a diagnostic test kit for the detection of hormone and/or the measuring of hormone levels in biological samples. Such a kit comprises necessary means of an immunoassay provided by the invention, such as (labelled) antibody, (hormone) conjugate, substrate, buffers or (coated) microtiterplates and/or instructions (often written on a accompanying leaflet or folder) to execute the method(s) provided by the invention. The invention is further explained in the experimental part below which cannot be seen as limiting the invention.

The invention further provides a method to preferentially protect small peptide or protein molecules against denaturation in mixtures comprising small and large (protein) molecules that are subject to a denaturation process. Denaturation of proteins is a multifaceted process which has properties to the good and to the bad. Denaturation occurs in any protein mixture that is being stored, even at low temperatures. The process can be enhanced or accelerated by raising temperatures or by adding denaturing agents. The invention provides a method whereby the speed of denaturation is influenced so that small protein molecules (peptides) denature less rapid than large molecules (polypeptides). A method provided by the invention comprises both heating and the addition of a denaturing agent. By selecting an appropriate combination, the combined action causes a large molecule to denature more rapidly than a small molecule. A method according to the invention is exemplified in the

experimental part, wherein the addition of a denaturing agent such as sodium salicylate combined with the heat treatment causes the large IGFBP molecule to denature more rapidly than the small IGF molecule, thereby leaving the small molecules relatively unaffected, allowing for example immunological detection of the IGF. Since denaturing of protein mixtures is a very common process, this principle can be utilised in numerous ways. For example, the invention provides a method wherein serum for use in cell-culture is denatured. Such sera are complex mixtures of small molecules, such as growth factors, some of which are beneficial for cell growth, and large molecules, such as complement, albumine, (immuno)globulines, some of which are detrimental for cell growth. Heat treatment, as is commonly used to prepare sera for cell-culture, denatures the beneficial small molecules just as well as the detrimental large molecules. By combining a heat treatment with a denaturing agent, such as sodium salicylate, said method preferentially denatures the larger molecules and relatively protects the small molecules; the resulting end product is now better suited for cell culture purposes than after heat treatment alone. Yet another method is provided wherein a denaturing agent is added to peptide/protein mixtures where by the peptide is better protected against denaturing processes. Such additions are beneficial in the production and/or storage of peptides, such as vaccines, hormones, and such. The invention also provides use of a denaturing agent, such as sodium salicylate, in a method provided by the invention.

Experimental part

Dressendörfer et al. [1] reported the development of an immunoassay for human salivary cortisol, using Streptavidin-Europium in conjunction with the DELFIA-system for time-resolved fluorometric end point measurement (TR-FIA). They found that this assay was working satisfactorily for human saliva samples, even if these were contaminated with up to 2% serum or plasma. However, with a substantially higher contamination (such as >3-5%) of plasma the assay is defective because of excessive BPP in the sample. Furthermore, as a consequence of our high demand for cortisol analyses in animal plasma or serum we have focused on a microtiterplate method suitable for conjugate. One objective of this study was to make the salivary assay suitable for measurement of cortisol, and other hormones (such as glucagon, insulin, gastrin, secretin, ACTH, LH, FSH, TSH, TRH, LHRH, vasopressin, IGF-I or II, EGF, somatotropin (growth hormone), prolactin, erytropoietin, EGF, cytokines, and others), in unextracted plasma or serum. Two problems were encountered during the cortisol . 1: Cortisol releasing agents [2,3,4,5,7,8], albeit reportedly suitable for human BPP, were not capable of eliminating the interference of for example bovine CBPP in the assay. 2: Streptavidin-Europium showed a high NSB that increased following a second coating with BSA.

Insulin-like growth factor I or II (IGF-I or II), related hormones, comprising for IGF-I a single-chain polypeptide with a molecular weight of 7649 daltons [22]), play an important role in growth and differentiation. The polypeptide is highly conserved as shown by the identical amino acid sequences of human, bovine and porcine IGF-I. In ovine IGF-I one amino acid is substituted [11]. At least six specific high-affinity IGF binding proteins (IGF-BPs) are present as binary and ternary complexes of approximately 30-50 and 150 kDa in the circulation and other body fluids and

frequently in high concentrations in cell culture media [16, 9, 23, 24, 18]. It is known that treatments like fasting, hypophysectomy or injection of somatotrophin induce changes in the plasma concentrations of different IGFBPs [12, 26, 20]. IGFBPs interfere in immunoassays for IGF-I and II and therefore their complete removal or inactivation after separation from IGF-I or II is necessary. So far several methods were used to separate IGF-I or II from its binding proteins before analysis including size exclusion chromatography at acidic pH, acid-ethanol and ethanol-acetone-acetic acid extraction, sometimes combined with cryo-precipitation or IGF-II coincubation, glycyl-glycine extraction and Sep-Pak chromatography. Acid size exclusion chromatography is considered to be the most optimal procedure, however, none of these methods completely removed or inactivated the IGFBPs [17, 27, 13, 14].

MATERIALS AND METHODS

Cortisol antiserum

Cortisol antiserum was raised by repetitive immunization of sheep with cortisol 3-(*o*-carboxymethyl)oxime:BSA.

IGF-I and antiserum

Human recombinant (E. coli) IGF-I (Boehringer, Mannheim, Germany, No. 1048066) was used for the preparation of tracer and standards. Lyophilized rabbit antiserum to IGF-I (UB2-495), 1.5-1.9% crossreactive with IGF-II, was provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Solutions of IGF-I and antiserum were stored at -80°C.

Preparation of steroid free bovine plasma

Heparin plasma was stirred with charcoal (50 mg/ml) at room temperature during the night and centrifuged at 12000g

for one hour. The steroid free plasma was diluted with water, using the same dilution factor as for samples, and traces of charcoal were removed by filtration through a 0.45 µm D26 filter (Sartorius, Göttingen, Germany, No. 6400204).

Sample pretreatment

Plasma or serum samples, that were stored at -20°C, were thawed, homogenized and centrifuged for 10 minutes at 1650g and 4°C. In the final assay procedure a sample was diluted 1:5 with water in a Safe-Lock tube (Eppendorf, Hamburg, Germany, No. 0030 121.023), homogenized and heated in a waterbath at predetermined conditions for example at 80°C for 30 minutes.

Pretreatment of sample and IGF-I standard dilutions

For IGF-I tests, all solutions were made in water purified by a Milli-Q/UF reagent grade water system (Millipore, Etten-Leur, The Netherlands, No. ZFMQ 240 U4). Sample and standard solutions were prepared in heat resistant 2 ml polypropylene micro tubes fitted with a lock-ring containing screw cap (Sarstedt, Nüm-brecht, Germany, No. 72693100). IGF-I was dissolved in 1 volume of 0.01N HCl followed by 9 volumes of 0.5M Na_2HPO_4, pH 7.4 to a stock concentration of 120 µg/ml. An aliquot of this stock solution was further diluted with water, containing 0.2% (w/v) bovine serum albumin (BSA, fraction V; Fluka AG, Buchs, Switzerland, No. 05480) to prepare standard solutions of IGF-I. A low IGF-I porcine heparin plasma to which IGF-I standard solutions were added was diluted with water to prepare solutions for recovery tests. A high IGF-I porcine heparin plasma was diluted with water to prepare solutions for linearity tests. To investigate the influence of blocking agents on IGF-I binding proteins 800 µl of these sample and standard solutions were diluted 1:1 with neutralized 0.4M trichloroacetic acid (TCA;

Merck, Darmstadt, Germany, No. 807), 2% (w/v) sodium salicylate (Sigma, St. Louis, USA, No. S-3007), 10mM benzamidine hydrochloride (Sigma, No. B-6506), neutralized 0.5% (w/v) 8-Anilino-1-naphthalenesulfonic acid (ANS; Sigma, No. A-1028), 4% (w/v) thimerosal (Sigma, No. T-5125) or water (control). Finally, 40 µl of these solutions contained 0.00, 0.01, 0.02, 0.04, 0.08, 0.12, 0.16, 0.24, 0.32, 0.48, 0.64, 1.28 and 2.56 ng IGF-I (standard solutions), 0.5 µl plasma and 0, 0.1 or 0.3 ng IGF-I (recovery test solutions), 0.5 and 1.0 µl plasma (linearity test solutions), respectively. To investigate the influence of temperature on IGF-I binding protein interference all plasma containing solutions of the TCA, sodium salicylate, benzamidine hydrochloride, ANS, thimerosal and water series were heated in a waterbath for 30 minutes at 20 (room temperature), 30, 40, 50, 60, 63, 66, 69, 72, 75, 78, 81, 84 or 87°C. Standards were not heated; replicate tubes of the 0.24 ng standard solution completed the same heating procedure as the plasma containing solutions to check for free IGF-I denaturation.

Assay procedures

The procedure of Dressendörfer et al. [1] was used with modifications. Water, purified by a Milli-Q/UF reagent grade water system (Millipore, Etten-Leur, The Netherlands, No. ZFMQ 240 U4) was used in all buffers and dilutions. Maxisorp microtiterplates (Nunc, Roskilde, Denmark, No. 437958A), stored at least one night at 4°C, were coated with 1600 ng of affinity purified rabbit anti-sheep immunoglobulin (Rockland, Gilbertsville, USA, No. 613-4102) per 200 µl 0.05M $NaHCO_3$, 0.02% (w/v) NaN_3, pH 9.6 during the night at 4°C, covered with a self-adherent foil to avoid evaporation. Plates were washed three times with 0.025M Na_2HPO_4, 0.05% (w/v) Tween 20, 0.025% (w/v) NaN_3, pH 7.5 in an automatic microtiter-plate washer (DELFIA platewash, Wallac Oy, No. 1296-026). Cortisol standard (Sigma, St. Louis, USA, No. H-5885) was solved in

ethanol (25 µg/ml); a 1:5 diluted steroid free bovine plasma was used to prepare concentrations of 0.00 (NSB), 0.00 (initial binding;B0), 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 ng/100 µl, which were pretreated following the same procedure as for sample dilutions. Duplicate 100 µl amounts of standard and sample dilutions were pipetted into the wells of the microtiterplate, followed by 50 µl cortisol-biotin stock solution (0.2 µg/ml ethanol) diluted 1:62.5 in double concentrated assay buffer [0.1M Tris-HCl, 0.3M NaCl, 0.1% (w/v) NaN_3, 0.02% (w/v) Tween 40, 15.74 mg/l diethylenetriaminepentaacetic acid, 0.1% (w/v) bovine γ globulin (Sigma, No. G-7516), pH 7.75, filtrated through a 0.45 µm filter], except NSB wells to which 50 µl ethanol diluted 1:62.5 in double concentrated assay buffer was added. Subsequently, 50 µl of sheep anti-cortisol serum diluted 1:75000 in double concentrated assay buffer was pipetted in all wells. The microtiterplate was shaken for 5 seconds (DELFIA plateshake, Wallac Oy, No. 1296-001), covered with foil and incubated during the night at ambient temperature. The plate was washed three times, followed by the addition of 200 µl of Eu-Labelled Streptavidin solution (Wallac Oy, No. 1244-360) diluted 1:1000 in single concentrated assay buffer and filtrated through 0.22 µm Millex-GV (Millipore, No. SLGV 025 BS). Incubation was allowed to proceed for 30 minutes at ambient temperature on a plate shaker. The plate was washed six times and 200 µl of "enhancement solution" (Wallac Oy, No. 1244-105) was added. After 15 minutes of incubation at ambient temperature on a plate shaker the plate was measured in a 1420 multilabel counter (Wallac Oy). Counting data were evaluated using Multicalc software (Wallac Oy). Samples with duplicate values showing more than 4% difference in relative binding ([B/B0] x 100%) were reanalyzed.

Release of cortisol from CBPP

Agent induced release:

Several agents were added to the cortisol-biotin incubation step with the following final concentration: 0.2M neutralized trichloroacetic acid [2] in combination with 2 mg/l danazol [3]; 1% (w/v) sodium salicylate [4,5].

Temperature induced release:

To investigate the influence of elevated temperatures on heparin or EDTA plasma or serum several samples were diluted 1:4 with two diluents, single concentrated assay buffer or water, and heated at various temperatures up to 90°C. If no solidification occured with a certain diluent the following experiment was carried out at a temperature of 90°C; if solidification occured at a certain temperature the following experiment with that diluent was carried out at a 10°C lower temperature. The diluents were used to prepare cortisol standards, cortisol standards in 1:4 diluted steroid free plasma and a 1:4 diluted sample with a high endogenous cortisol concentration that was diluted further with 1:4 diluted steroid free plasma. Each series of standard and sample dilutions was heated for 15-30 minutes at the chosen temperature. Amounts of 100 µl of all series were tested in the FIA. The procedure that was chosen on the basis of these results was further optimized with respect to dilution factor and heating temperature. Finally, the influence of increasing amounts of not inactivated cortisol binding globulin (CBG) on the assay was demonstrated. Human CBG (MW 55700; UCB, Braine L'Alleud, Belgium, No. H097/H) was used for lack of pure bovine CBG.

Influence of BSA and heparin on NSB

Two plates were coated according to the normal procedure. A second coating step with 300 µl of 1% (w/v) BSA (Boehringer, Mannheim, Germany, No. 735094) in single concentrated assay buffer was performed during one hour at

room temperature with one of the plates. The cortisol-biotin and Streptavidin-Europium incubations were performed in assay buffer containing 0.5% (w/v) BSA. NSB of both plates were compared.

The NSB of pretreated steroid free heparin plasma was tested for final BSA concentrations of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 0% (w/v) in combination with 0, 10 and 25 IU of heparin (LEO, Weesp, The Netherlands) added per well during the cortisol-biotin and the Streptavidin-Europium incubation.

The NSB of serum, EDTA and heparin plasma samples (all after pretreatment) was tested with final BSA concentrations of 1/2 or 0% (w/v) during the cortisol-biotin and the Streptavidin-Europium incubation.

The effect of replacing BSA by gelatin (Sigma No. G-9391) was investigated. A second coating step with 0.5% (w/v) gelatin in single concentrated assay buffer during one hour at room temperature was performed in combination with a final 0.1% (w/v) concentration during the cortisol-biotin and the Streptavidin-Europium incubation.

Assay validation

Specificity of the sheep anti-C3-CMO:BSA serum towards potentially crossreactive steroids was determined. Sensitivity of the assay was calculated as the detection limit, defined by Abraham [7] as the antigen concentration corresponding to the lower confidence limit of the initial binding (B0-2 sd). The B0 was determined in 18-fold. Linearity was checked by measurement of pretreated heparin and EDTA blood plasma samples serially diluted with pretreated steroid free plasma. Recovery of several known amounts of standard cortisol, added to samples before pretreatment, was determined. Three control samples, a high heparin plasma sample, a low serum sample and a 1:1 mixture of both were analyzed in each assay to check for linearity

and to determine the inter-assay reproducibility. These samples were also used to determine the intra-assay reproducibility.

Iodination of IGF-I and radioimmunoassay

The labelling procedure of Thorell & Johansson [25] was used with minor modifications. The following reactants were added to the vial containing 1 mCi $Na^{125}I$ in 10 µl alkaline solution (The Radiochemical Centre, Amersham, England, No. IMS30): 4 µg IGF-I in 33.3 µl stock solution; 2 µg lactoperoxidase (Sigma, No. L2005) in 5 µl water; 10 µl 44µM H_2O_2 (Merck, No. 107209). The contents of the vial were mixed slowly for 75 seconds. The reaction was stopped by the addition of 100 µl of cold (4°C) radioimmunoassay (RIA) buffer (0.05M Na_2HPO_4, 0.15M NaCl, 0.1% (w/v) NaN_3, pH 7.2) followed by 200 µl of cold RIA buffer containing 0.5% (w/v) BSA. Purification of labelled IGF-I and radioimmunoassay procedures were performed as described for ovine follicle-stimulating hormone [10] with minor modifications. Purification: labelled IGF-I was separated from free ^{125}I using a 30 x 0.9 cm column of Sephadex G25-coarse (Pharmacia Fine Chemicals, Uppsala, Sweden, No. 17003401) and 50 fractions of 0.5 ml were collected; to separate immunoreactive labelled IGF-I from other material a 90 x 1.6 cm column of Sephadex G50-coarse (Pharmacia No. 17004401) was used and 130 fractions of 1 ml were collected; displacement of labelled IGF-I of the fractions was tested with 0.24 ng unlabelled IGF-I. Radioimmunoassay: the lyophilized antiserum was reconstituted to its original volume with water and 50 µl of a 1:1800 dilution was used to give an initial binding of the label of ~40%; 50 µl of tracer, containing 16000 cpm of [^{125}I]IGF-I, was added; 40 µl of standard or sample dilution (see: Pretreatment of sample and IGF-I standard dilutions) was pipetted; total volume during the first antibody incubation was 500 µl; all measurements were done in triplicate.

RESULTS

Release of cortisol from CBPP

Agent induced release:

In a binding experiment without plasma the use of neutralized trichloroacetic acid in combination with danazol lowered the B0 in buffer to 80% of the normal value. An equal effect was observed with sodium salicylate. Fig.1 shows the results for the TCA/danazol combination. The steroid free plasma caused a severe reduction of the B0. In the presence of sodium salicylate the steroid free plasma inhibited the B0 completely (data not shown). Temperature induced release: In plasma samples diluted with single concentrated assay buffer solidification, caused by protein precipitation, occurred at a temperature of about 70°C. Fig.2 shows the results for buffer diluted series that were heated at a temperature of 60°C. Heating at this temperature does not prevent the steroid free plasma from reducing the B0 considerably. Plasma samples diluted with water showed no solidification at all tested temperatures. Sometimes individual samples were less clear following heating; in these cases 10 minutes of centrifugation at 1650g could not restore clearness and no sediment was formed. Validation experiments performed with these samples showed no divergent results. Fig.3 shows the results for the water diluted series that were heated at a temperature of 90°C. There is only a minor difference between the B0's of the standard curves in water and steroid free plasma. The sample dilution curve shows good parallellity with the standard curves. The reading of both high and low samples in the accurate part of the standard curve was accomplished in the final assay procedure by analysing 100 µl of a 1:5 instead of a 1:4 dilution of sample with water. Fig.4 shows the initial binding of 1:5 diluted steroid free bovine plasma heated for 30 minutes at different

temperatures. Heating at 80°C was chosen for the assay procedure in the subsequent experiments. Fig.5 demonstrates the effect on the B0 of several amounts of human CBG added to steroid free heparin plasma after sample pretreatment. Fig. 7 shows the initial binding of 1:5 diluted steroid free human, chicken, rabbit and porcine plasma heated at various temperatures.

Influence of BSA and heparin on NSB

After a second coating step with BSA the non-specific binding increased to approximately 10 times the value of the single coating step procedure. We, however, observed during the experiments that NSB was partly reduced if heparin containing samples were analysed. Fig.6 shows the influence of several combinations of BSA and heparin in the assay buffer on the NSB of pretreated steroid free heparin plasma. Table 1 shows the NSB of different sample types, using final BSA concentrations of 0 and 0.5% (w/v). The use of BSA necessitates individual NSB correction for all samples. With BSA free buffer NSB is equal for all samples. The NSB levels of tabel 1 are roughly a factor 2 lower compared to corresponding data (0 or 0.5% BSA, no heparin added) of fig. 6. We have found this to be due to the use of different batches of reagents. With BSA free buffer, however, the absolute differences in NSB are to small to affect the assay. The use of gelatin during a second coating step and in the assay buffer resulted in a NSB equal to BSA free buffer. There was no influence of gelatin on other validation parameters of the assay (data not shown).

Assay validation

Specificity of the sheep anti-C3-CMO:BSA serum is shown in table 2. Detection limit was 0.01 ng per well, corresponding to 0.5 ng cortisol/ml if 100 µl of a pretreated

1:5 blood plasma dilution was taken for analysis. Results for linearity and recovery are shown in tables 3 and 4, respectively. Mean concentrations, inter- and intra-assay coefficients of variation of the three control samples were 71.1, 39.2 and 10.3 ng/ml, 7.3, 9.0 and 11.2% (n = 73), 8.2, 7.9 and 11.3% (n = 16), respectively.

IGF-I assay

The determined values of endogenous and exogenous IGF-I of the recovery test samples, the linearity test samples and the standard denaturation check sample, incubated at different temperatures in a TCA or sodium salicylate (SS) solution or water are shown in figures 8 to 10. All values were read in the accurate part of the standard curves (between 20 and 80% of the initial binding [B0]); standard curves matched exactly (curves not shown).

These results clearly demonstrate that the use of blocking agents for CBPP in the assay buffer cannot prevent a drastic decrease of antibody bound cortisol-biotin. A similar behaviour of cortisol-biotin towards another blocking agent, 8-anilino-1-naphthelene sulfonic acid, was reported by Dressendörfer et al. [1]. A final concentration of 1% (w/v) of this chemical was not capable of displacing significant amounts of cortisol-biotin from CBG. The use of buffer for the dilution of samples limited the denaturation temperature to 60°C. This temperature was shown to be suboptimal for the denaturation of human CBG; furthermore, our results show that for for example bovine CBG a higher temperature is necessary. If cortisol standards prepared in water or in water diluted steroid free plasma were heated at 90°C, the standard curves were nearly identical. The small difference between the curves is not relevant for the assay as both standards and unknowns contain an equal amount of plasma. During optimization of the procedure a heating temperature of 80°C

was shown to be sufficient for the complete inactivation of CBPP in bovine plasma and a sample dilution factor 5 ensured the reading of both low and high samples in the accurate part of the standard curve, however, it is clear that other dilutions can be chosen as well. During the first antibody incubation addition of 8 ng/well (0.7 nM) human CBG resulted in a significant decrease of the B0. Sharma et al. [8] reported a 60 nM CBG serum concentration for bovine calves, resulting in a 6 nM concentration in the assay. From these data the necessity of a complete inactivation of CBG is obvious. The high NSB of Streptavidin-Europium in the absence of cortisol-biotin shows that it is caused directly by the former and not indirectly by Streptavidin-Europium bound to nonspecifically bound cortisol-biotin. The increase of the NSB following a second coating with BSA demonstrates that Streptavidin-Europium is not bound to the microtiter wall directly but via BSA. Since the NSB appeared to be proportional to the BSA concentration in the buffer the problem could be solved simply by omitting the use of BSA. Heparin clearly competes with Streptavidin-Europium for the binding sites on BSA, which is also the explanation for the observed lower NSB of heparin plasma compared to EDTA plasma. It was shown that the ability to bind Streptavidin-Europium non-specifically is not a general property of proteins, since bovine γ-globulin, which is present in the assay buffer, and gelatin lack this behaviour. The use of a BSA free assay buffer resulted in a very low NSB level that was not influenced anymore by the different sample types, making individual corrections redundant. Since the Biotin-Streptavidin-Europium system can be used in an extensive range of laboratory tests, the removal of BSA from procedures which are based on specific measurement of Streptavidin-Europium fluorescence is beneficial to a great variety of tests beyond mere steroid hormone tests.

In the case of the IGF-I assays the results showed that heating alone is not optimal to inactivate the IGFBPs.

Compared to the high affinity IGFBPs, steroid binding proteins have a low affinity for their target, which might explain the observed difference. In water at a temperature of 87°C about 40% of the 0.24 ng IGF-I of the control solution was denaturated, but under the same circumstances no influence was observed on the results of the linearity plasma, which were rather constant over the entire temperature range and even showed good linearity. In this situation, however, linearity is not a sufficient criterion for the correctness of the results. Several groups have reported that IGFBPs present in extracted sera may interfere with the assay despite the fact that displacement curves for these extracted sera are parallel to the standard curve. It was supposed that competition of IGFBPs for binding of either labelled or unlabelled IGF-I is involved [15, 19, 21, 13]. If SS or TCA were used in the solvent, results for the linearity plasma changed at 60 or 63°C, respectively. In case of SS recoveries in the temperature range from 72°C and higher were very near to 100% and no denaturation of free IGF-I occurred. With TCA, recoveries in the same temperature range showed a little more variation and denaturation of free IGF-I started at a temperature of approximately 84°C. So, surprisingly, denaturing agents such as SS or TCA appeared to protect the free IGF-I from denaturation at higher temperatures. Linearity was perfect for both agents at temperatures higher than 63°C. Within the SS temperature range with optimal recoveries (72°C and higher) the small differences in the I-GF-I concentrations of the linearity plasma were not significant. So the use of SS at a temperature between 72 and 87°C seems to be a good choice.

Correlation of a method according to the invention with acid size exclusion chromatography (ASEC) for IGF-I.

Materials

Water purified by a Milli-Q/UF reagent grade water system (Millipore, Etten-Leur, The Netherlands, No. ZFMQ 240 U4) was used in all procedures. Chemicals, if not further specified, were of p.a. quality.

Blood plasma samples

Blood samples, taken from female and castrated male pigs at an age of 69 to 431 days, were collected in heparinized tubes. Blood plasma was stored at -20°C.

Iodination of IGF-I and radioimmunoassay

Lyophilized rabbit antiserum to IGF-I (UB2-495), 1.5-1.9% crossreactive with IGF-II, was provided by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, USA and was reconstituted to its original volume with water. Human recombinant (E. coli) IGF-I (Boehringer, Mannheim, Germany, No. 1048066) was used for the preparation of tracer and standards. IGF-I was dissolved in 1 volume of 0.01N HCl followed by 9 volumes of 0.5M Na_2HPO_4, pH 7.4 to a stock concentration of 120 µg/ml. Stock solutions of antiserum and IGF-I were stored at -80°C. The labelling procedure of Thorell & Johansson (1971) was used with minor modifications. The following reactants were added to the vial containing 1 mCi $Na^{125}I$ in 10 µl alkaline solution (The Radiochemical Centre, Amersham, England, No. IMS30): 4 µg IGF-I in 33.3 µl stock solution; 2 µg lactoperoxidase (Sigma, St. Louis, USA, No. L2005) in 5 µl water; 10 µl 44µM H_2O_2 (Merck, Darmstadt, Germany, No. 107209). The contents of the vial were mixed slowly for 75 seconds. The reaction was stopped by the addition of 100 µl of cold (4°C) radioimmunoassay (RIA) buffer (0.05M Na_2HPO_4, 0.15M NaCl, 0.1% (w/v) NaN_3, pH 7.2) followed by 200 µl of cold RIA buffer containing 0.125% (w/v) bovine serum albumin (BSA, essentially globulin free; Sigma, No. A7638) showing no detectable IGF-I or IGFBP contamination in all batches used

(RIA buffer 0.125% BSA). Purification of labelled IGF-I and radioimmunoassay procedures were performed as described for ovine follicle-stimulating hormone (Erkens et al., 1992) with minor modifications. Shortly: Labelled IGF-I was separated from free ^{125}I using a 30 x 0.9 cm column of Sephadex G25-coarse (Pharmacia Fine Chemicals, Uppsala, Sweden, No. 17003-401) and 50 fractions of 0.5 ml were collected; to separate immunoreactive labelled IGF-I from other material a 90 x 1.6 cm column of Sephadex G50-coarse (Pharmacia No. 17004401) was used and 130 fractions of 1 ml were collected. Both columns were eluted at 4°C with RIA buffer 0.125% BSA. Fractions were tested for antibody binding in both presence and absence of 0.24 ng unlabelled IGF-I and for non-specific binding. Fractions showing the best immunoreactivity were pooled and stored at 4°C. RIA: IGF-I standard concentrations of 0.00 (initial binding; B0), 0.01, 0.02, 0.04, 0.08, 0.12, 0.16, 0.24, 0.32, 0.64, 1.28 and 2.56 ng/40 µl were prepared by diluting an aliquot of the IGF-I stock solution with 0.1% (w/v) BSA in water. Standard tubes were always adjusted to the same chemical composition as the tubes containing the sample dilutions from various pretreatments. RIA buffer 0.125% BSA was used to dilute antiserum and tracer and to achieve a total volume of 500 µl. Fifty µl of tracer, containing 16000 cpm of [^{125}I]IGF-I, and 50 µl 1:1800 diluted antiserum were added to give a B0 of ~40%. Following a 6 days incubation time at 4°C 1 ml of a water/ice cooled 1:8 dilution of donkey anti-rabbit solid phase (IDS, Boldon, England, No. AA-SAC1) in RIA buffer 0.1% BSA was added. Before use the solid phase suspension was washed twice with an equal volume of RIA buffer 0.1% BSA to remove possibly IGF-I or IGFBP contaminated BSA. After a one night incubation at 4°C the tubes were centrifugated for 5 minutes at 1650g and 4°C, after which the supernatants were aspirated until a volume of ~0.4 ml was left. The precipitates were washed twice with 2.2 ml water/ice cooled RIA buffer and were counted for 5 minutes. Measurements were done in triplicate.

Heat pretreatment of diluted blood plasma with or without potential blocking agents for IGFBP's.

Trichloroacetic acid (Eskola et al., 1985), sodium salicylate (Kane, 1979; Pourfarzaneh et al., 1980), 8-Anilino-1-naphthalenesulfonic acid and thimerosal (Kane, 1979), all of which were reported as blocking agents for steroid and/or thyroid hormone binding plasma proteins, were chosen as potential blocking agents for IGFBP's. The following diluents were made: neutralized 0.2M trichloroacetic acid (TCA; Merck, No. 807), 1% (w/v) sodium salicylate (SOSA; Sigma, No. S-3007), neutralized 0.25% (w/v) 8-Anilino-1-naphthalene-sulfonic acid (ANS; Sigma, No. A-1028) and 2% (w/v) thimerosal (THIM; Sigma, No. T-5125). For linearity and recovery testing 20 and 40 µl of a sample with a high IGF-I concentration and 20 µl of a sample with a low IGF-I concentration, to which 0, 4 or 12 ng of IGF-I standard were added, were diluted to a volume of 1600 µl, using one of the diluents or water. Aliquots of these solutions were heated for 30 min in a waterbath at temperatures of 20, 30, 40, 50, 60, 63, 66, 69, 72, 75, 78, 81, 84 or 87°C in heat resistant 2 ml polypropylene micro tubes fitted with a lock-ring containing screw cap (Sarstedt, Nümbrecht, Germany, No. 72693100). IGF-I standard, diluted with one of the diluents or water to a concentration of 0.24 ng/40 µl, completed the same heating procedure as the plasma containing solutions to check for free IGF-I denaturation (standard denaturation check sample; SDC sample). Following heating all solutions were cooled to ambient temperature and 40 µl aliquots, containing 0.5 or 1.0 µl of linearity plasma, 0.5 µl of recovery plasma with 0, 0.1 or 0.3 ng of IGF-I standard or 0.24 ng of IGF-I standard without plasma, were analyzed by RIA. On the basis of these results the SOSA experiment was extended with temperatures of 90, 93 and 96°C.

Acid size exclusion chromatography

Ten blood plasma samples with a range of IGF-I concentrations, as determined after heat pretreatment in SOSA at 78°C, were chromatographed at 4°C on the column of Sephadex G50-coarse used for the purification of labelled IGF-I. 1M Acetic acid, containing 0.2% (w/v) BSA, was used to wash and equilibrate the column and to elute the samples. A sample was mixed with an equal volume of 2M acetic acid, 0.4% (w/v) BSA and was left at room temperature for a few hours. Four hundred µl of this mixture was applied to the column and 175 fractions of 1 ml were collected at a speed of one fraction per 90 sec. The precise fraction volume was checked for two tubes of each collection on the basis of weight measurements and fractions were neutralized with an accurately known amount of 10M NaOH. In order to check the recovery of labelled and unlabelled IGF-I of this procedure, one of the samples was chromatographed with and without the combined addition of 63000 cpm of ^{125}I-IGF-I and 320 ng of standard IGF-I. After the addition the sample was left at room temperature for a few hours followed by the normal procedure. Forty µl amounts of all the fractions of both experiments were analyzed by RIA. On the basis of these results the range of fractions containing the IGF-I or the IGFBP's were determined and an accurate part of the expected IGF-I containing fractions of the other samples was mixed and analyzed by RIA. This was done to reduce the number of analyses per sample and to avoid the inaccurate measurement of low IGF-I fractions at the beginning and the end of the peak. Individual fractions preceding and following a mixture were analyzed as controls for the mixing procedure. Depending on the expected level of the IGF-I concentration of a mixture, amounts of 40 to 400 µl were taken for analysis. Results of SOSA pretreatment and ASEC were correlated.

Validation of the radioimmunoassay

Detection limit of the assay, defined by Abraham (1974) as the antigen concentration corresponding to the lower confidence limit of the number of counts specifically bound in the absence of unlabelled antigen (B0-2sd), was 19 pg per tube.

Porcine blood plasma control sample amounts of 1, 7/8, 3/4, 5/8, 1/2, 3/8, 1/4 and 1/8 µl, that were analyzed during 8 assays, yielded the following mean IGF-I concentrations and inter-assay CVs: 481, 469, 472, 467, 470, 465, 447 and 460 ng/ml and 10.7, 8.6, 9.6, 8.4, 8.7, 9.7, 10.6 and 11.8%, respectively. The intra-assay CV for 12 replicates of the 1/2 µl amount during these assays was 5.0 ± 1.8% (mean and sd).

For data on recovery of standard IGF-I added to samples before various pretreatments see: results of these pretreatments.

Heat pretreatment

The determined values of endogenous and exogenous IGF-I of the recovery test samples, the linearity test samples and the SDC sample, incubated at different temperatures in water, TCA or SOSA are shown in figures 8 to 10. The use of TCA and SOSA showed no influence on the RIA. Standard curves matched exactly (curves not shown). If ANS and THIM were used the IGF-I concentration of the SDC sample at 63°C decreased to a value of 35 and 83% of the control value, respectively, and recoveries were extremely bad. Further, the use of ANS and THIM resulted in a shift of the standard curve to the right, making the assay less sensitive (results not shown).

Acid size exclusion chromatography

The chromatograms of a sample with and without the combined addition of labelled and unlabelled IGF-I are shown in figure 11. For the sake of convenience the decreasing RIA B0, caused by increasing amounts of IGFBP's, was calculated and represented as apparent IGF-I concentration (left peak). Amounts

of 40 to 400 µl of the eluent showed no influence on the B0 of the RIA. Eight different amounts, ranging between 20 and 160 µl, of a mixture of IGF-I peak fractions of the native plasma showed perfect linearity. Seven different amounts, ranging between 40 and 160 µl, of a mixture of IGFBP peak fractions of the native plasma showed no linearity at all; the apparent IGF-I concentration increased from 0.93 to 6.89 ng/ml. Recovery of exogenous unlabelled IGF-I was 84.6%. Labelled IGF-I was eluted from the column completely (100.9% if compared to the same amount of unchromatographed tracer) The small peak of labelled IGF-I, coinciding with the IGFBP fractions, represents aggregated label. Repurification of the tracer showed an increase of this peak during aging. Controls to check for the correct mixture of ASEC fractions were all near or below the detection limit of the assay.

Correlation between SOSA and ASEC results

Fig. 12 shows the IGF-I concentrations and Pearson correlation of 10 plasma samples determined after SOSA pretreatment at 78°C and following ASEC.

Surprisingly, SOSA and also TCA appeared to protect the IGF-I in the SDC sample from denaturation at temperatures above 75°C. Between 72 and 84°C linearity and recovery with SOSA and TCA were good, but recovery was slightly better for SOSA. Therefore SOSA at 78°C was chosen as pretreatment procedure. Validation parameters for the RIA in combination with this procedure were excellent. Correlation of results of a method according to the invention with ASEC, was 0.92 (n=10, $p<0.001$). However, in contrast with a method as provided by the invention, an incomplete IGF-I recovery following ASEC was demonstrated. Results after SOSA pretreatment and ASEC were thus highly correlated, however, ASEC showed a number of major drawbacks. Besides the extremely laborious chromatography procedure ASEC resulted in an incomplete recovery of standard IGF-I added to plasma. For seven out of ten plasma

samples the determined endogenous IGF-I concentration after ASEC was substantially lower compared to SOSA pretreatment. It remained unclear whether these discrepancies were caused by acidification, chromatography or the combination of these procedures. In conclusion: the method for the analysis of IGF-I in porcine plasma, using heat treatment in combination with a denaturing or blocking agent, yielded reliable results after a nonlaborious procedure, suitable for the simultaneous analysis of high numbers of samples. Besides IGF-II, sharing the same IGFBP's with IGF-I, many biomolecules and their sufficiently more or less temperature sensitive binding partner(s) are clear candidates for selective heat inactivation followed by quantitative measurement of the unaffected counterpart(s).

Reactivation of IGFBP's after SOSA pretreatment

Pretreated porcine plasma was acidified according to the ASEC procedure, followed by a two hrs incubation at room temperature. Labelled IGF-I was added, the solution was neutralised and chromatographed at neutral pH. Reactivation of IGFBP's was demonstrated by the presence of a radioactive peak in the fractions with the IGF-I/IGFBP complex molecular weight range.

Legends to the figures

Fig. 1. Influence of TCA/Danazol on steroid binding plasma protein interference.
Standards (30 µl) were prepared in buffer or in steroid free plasma (SFP). A sample (30 µl) was diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 with SFP (axis not shown). Final danazol and neutralized trichloroacetic acid concentrations during the cortisol-biotin incubation were 2 mg/l and 0.2M, respectively.

Fig. 2. Influence of buffer/60°C on steroid binding plasma protein interference.
Standards (100 µl) were prepared in buffer or in a 1:4 dilution of steroid free plasma (SFP) in buffer. A sample was diluted 1:4 with buffer (100 µl) and further diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) with a 1:4 dilution of SFP in buffer (axis not shown). Before use in the FIA standard and sample dilutions were heated at 60°C for 30 minutes, followed by cooling to ambient temperature.

Fig. 3. Influence of water/90°C on steroid binding plasma protein interference.
Standards (100 µl) were prepared in water or in a 1:4 dilution of steroid free plasma (SFP) in water. A sample was diluted 1:4 with water (100µl) and further diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) with a 1:4 dilution of SFP in water (axis not shown). Before use in the FIA standard and sample dilutions were heated at 90°C for 30 minutes, followed by cooling to ambient temperature.

Fig. 4. Effect of pretreatment temperature on B0 of steroid free plasma.
Steroid free bovine plasma was diluted 1:5 with water and heated at several temperatures for 30 minutes, followed by cooling to ambient temperature. Amounts of 100 µl were tested in the FIA. Vertical bars show positive standard deviations (missing bars were smaller than the marker size).

Fig 5. Effect of uninactivated hCBG on the B0 of pretreated steroid free plasma.
Uninactivated human CBG amounts, ranging from 0 to 5000 ng, were tested in the FIA in combination with 100 µl of a 1:5 dilution of steroid free plasma in water that was heated at 80°C for 30 minutes, followed by cooling to ambient temperature.

Fig. 6. NBS of pretreated steroid free plasma in relation to BSA / Heparin concentration.
Steroid free plasma was diluted 1:5 with water and heated at 80°C for 30 minutes, followed by cooling to ambient temperature. NSB of 100 µl amounts was tested with several combinations of BSA and heparin during the cortisol-biotin and Streptavidin-Europium incubation.

Fig. 7. Effect of pretreatment temperature on B0 of steroid free plasma of other species.
Steroid free human, chicken, rabbit and porcine plasma was diluted 1:5 with water and heated at several temperatures for 30 minutes, followed by cooling to ambient temperature.
Amounts of 100 µl were tested in the FIA. Vertical bars show positive standard deviations (missing bars were smaller than the marker size).

Fig 8. to 10. IGF-I asays.
Right Y-axis: (+) ng IGF-I/µl recovery plasma (0.5 µl analyzed); (O) ng IGF-I/µl linearity plasma (0.5 µl analyzed);

( ) ng IGF-I/μl linearity plasma (1.0 μl analyzed); (□) ng IGF-I standard.
Left Y-axis: (⮝) % recovery of 0.1 ng IGF-I standard added to 0.5 μl recovery plasma; (▲) % recovery of 0.3 ng IGF-I standard added to 0.5 μl recovery plasma.
Upper dotted line shows the preheating value (0.24 ng) of the standard denaturation check sample; lower dotted line represents the 100% recovery level.

Fig 11.
Acid size exclusion chromatography of 200 μl heparinized porcine blood plasma with (Ò) or without (●) the combined addition of 320 ng of IGF-I standard and 63000 cpm of ^{125}I-IGF-I (+) on a 90 x 1.6 cm column of Sephadex G50-coarse. Radioactivity per total (1 ml) fraction was measured directly (right Y-axis). The amount of endogenous and exogenous unlabelled IGF-I per 40 μl fraction volume was determined by radioimmuno-assay (left Y-axis). The maximal amount of chromatographed ^{125}I-IGF-I in the assay was 0.75% of the tracer activity and was not corrected for. For convenience the amount of IGFBP (left peak) was calculated as apparent IGF-I concentration by means of the reduced initial binding in the radioimmunoassay. The small ^{125}I-IGF-I peak coinciding with the IGFBP fractions represents aggregated label. This peak was found to increase during aging of the tracer.

Fig. 12:
Relationship and Pearson correlation of IGF-I concentrations, determined by radioimmunoassay, of ten heparinized porcine blood plasma samples following acid size exclusion chromatography (ASEC) or heat treatment at 78°C in a 1% (w/v) sodium salicylate dilution (SOSA).

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CLAIMS

1. A method for determining the presence of a hormone in a sample, said sample possibly containing a binding protein for said hormone, comprising reducing the binding between said hormone binding protein and said hormone by heating said sample and further comprising detecting the presence of the hormone not-bound to said binding protein.

2. A method according to claim 1 wherein the binding protein is a binding plasma protein, such as a sex hormone binding globulin (SHBG) or an insulin-like growth factor binding protein (IGFBP).

3. A method according to claim 1 or 2 wherein said sample is pre-heated.

4. A method according to claim 1, 2 or 3 wherein the binding protein is denatured more rapidly than the hormone.

5. A method according to any of claims 1 to 4 wherein the hormone is a steroid hormone, such as cortisol, or a small peptide hormone, such as insuline-like growth factor.

6. A method according to any of claim 1 to 5 which comprises heating a biological sample, such as cell culture fluid, saliva or urine, preferably serum or plasma.

7. A method according to claim 6 which additionally comprises cooling said sample to ambient temperature after heating.

8. A method according to any of above claims which additionally comprises adding to said sample a denaturing agent, such as sodium salicylate or trichloroacetic acid.

9. A method according to claim 7 or 8 which additionally comprises diluting said sample with water.

10. A method according to claim 6, 7 or 8 wherein said sample is derived from a captured or domesticated animal.

11. A method according to any of above claims further comprising using an immunoassay.

12. A method according to claim 11 further comprising reducing non-specific background staining found with an

immunoassay using (strept)avidin by at least significantly reducing the use of BSA and/or by using heparin in said immunoassay.

13. A method according to claim 11 wherein the immunoassay is a (time-resolved) fluoroimmunoassay using (strept)avidin labelled Europium.

14. An assay which is capable of measuring a hormone level in a biological sample which sample substantially contains a specific binding plasma protein reactive with said hormone using a method according to any of claims 1 to 13.

15. An assay according to claim 14 wherein the hormone is cortisol or insulin-like growth factor.

16. An assay according to claim 14 or 15 wherein the sample is derived from a captured or domesticated animal.

17. An assay according to any of claims 14 to 16 wherein the sample is plasma or serum.

18. An assay according to any of claims 14 to 17 which is an immunoassay, preferably an enzyme-linked immunoassay or a (time-resolved) fluoroimmunoassay.

19. A diagnostic kit which comprises an instruction to execute a method according to any one of claims 1 to 13 and/or comprises necessary means for an assay according to any one of claims 14 to 18.

20. Use of a method according to any one of claims 1 to 13 in an assay to detect the presence of and/or determine the level of a hormone in a sample.

21. Use according to claim 20 wherein the hormone is cortisol or insulin-like growth factor.

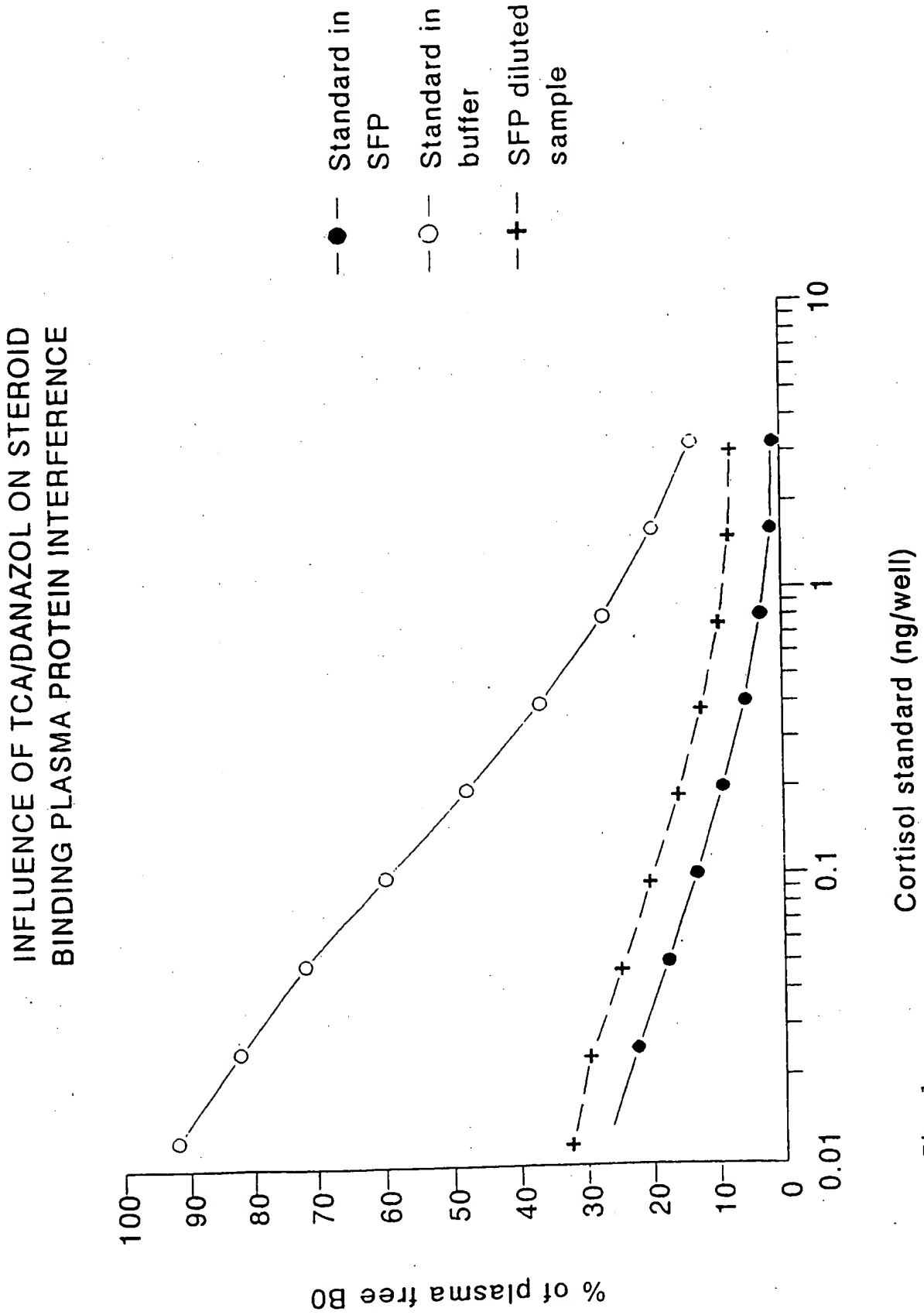
INFLUENCE OF TCA/DANAZOL ON STEROID
BINDING PLASMA PROTEIN INTERFERENCE
Standard in SFP
Standard in buffer
SFP diluted sample
% of plasma free B0
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
Cortisol standard (ng/well)

Fig. 1

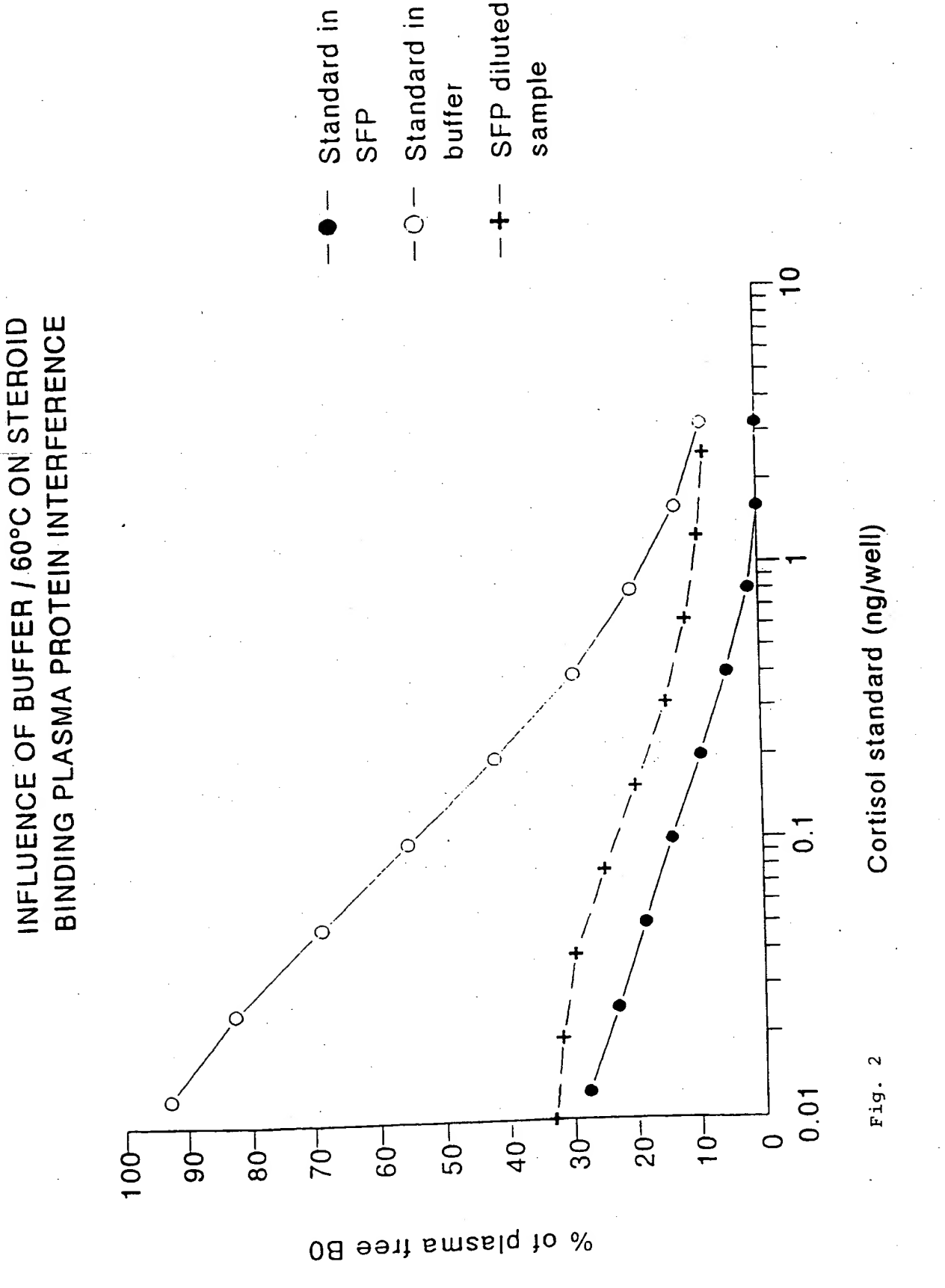
INFLUENCE OF BUFFER / 60°C ON STEROID
BINDING PLASMA PROTEIN INTERFERENCE
Standard in SFP
Standard in buffer
SFP diluted sample
% of plasma free Bo
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
Cortisol standard (ng/well)

Fig. 2

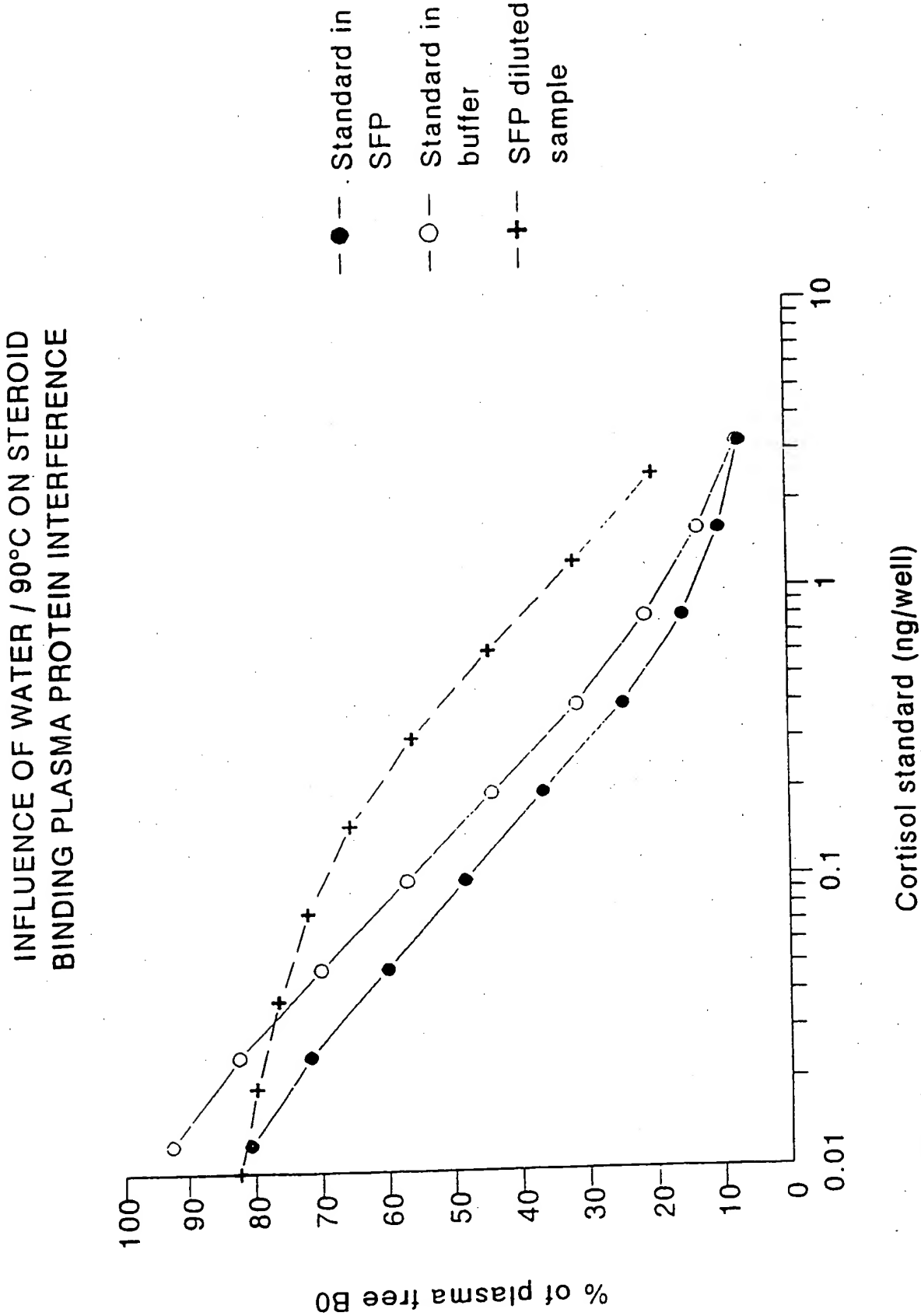
INFLUENCE OF WATER / 90°C ON STEROID
BINDING PLASMA PROTEIN INTERFERENCE
Standard in SFP
Standard in buffer
SFP diluted sample
% of plasma free B0
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
Cortisol standard (ng/well)

Fig. 3

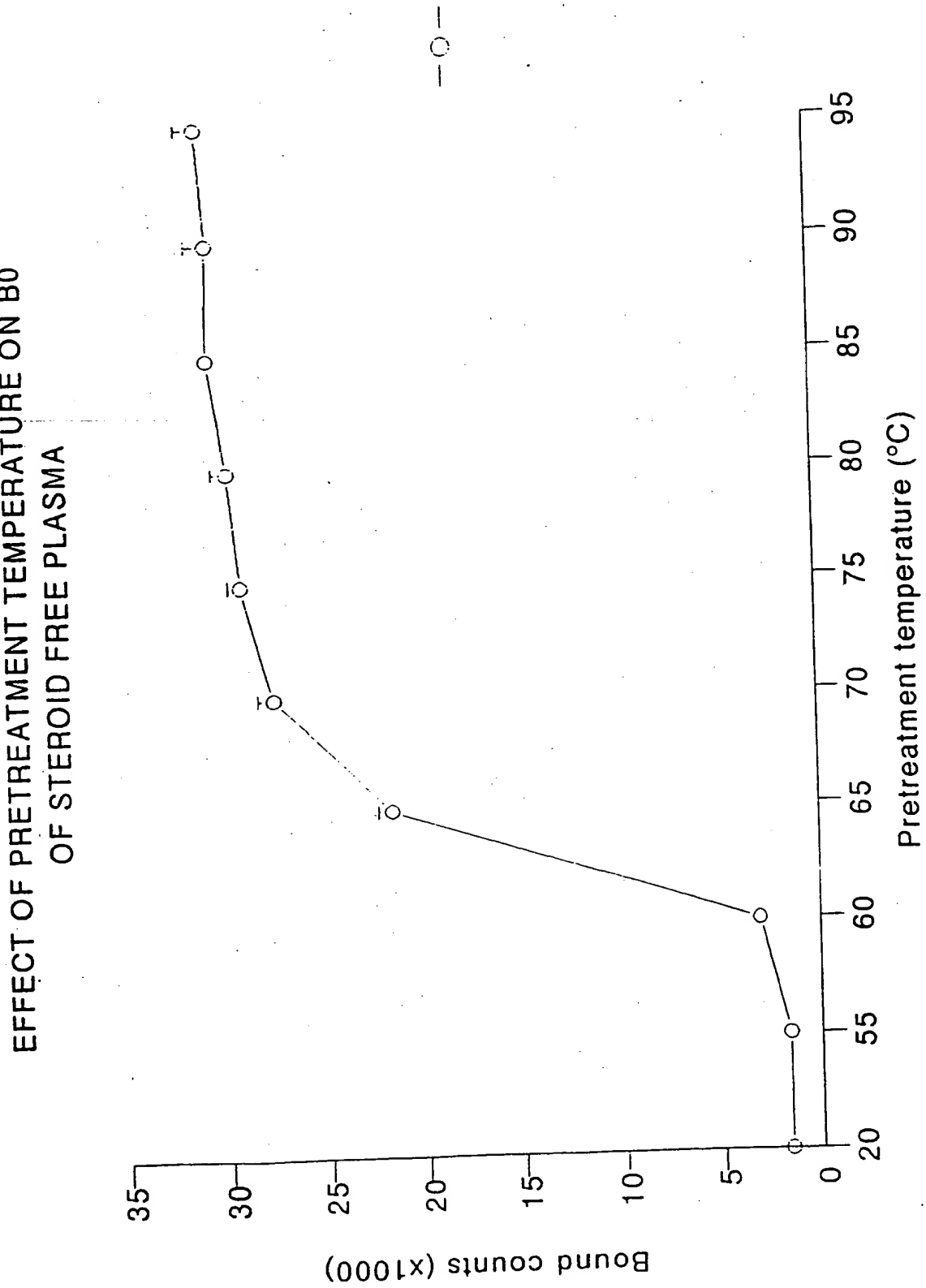
EFFECT OF PRETREATMENT TEMPERATURE ON B0
OF STEROID FREE PLASMA
Bound counts (x1000)
0
5
10
15
20
25
30
35
20
55
60
65
70
75
80
85
90
95
Pretreatment temperature (°C)

Fig. 4

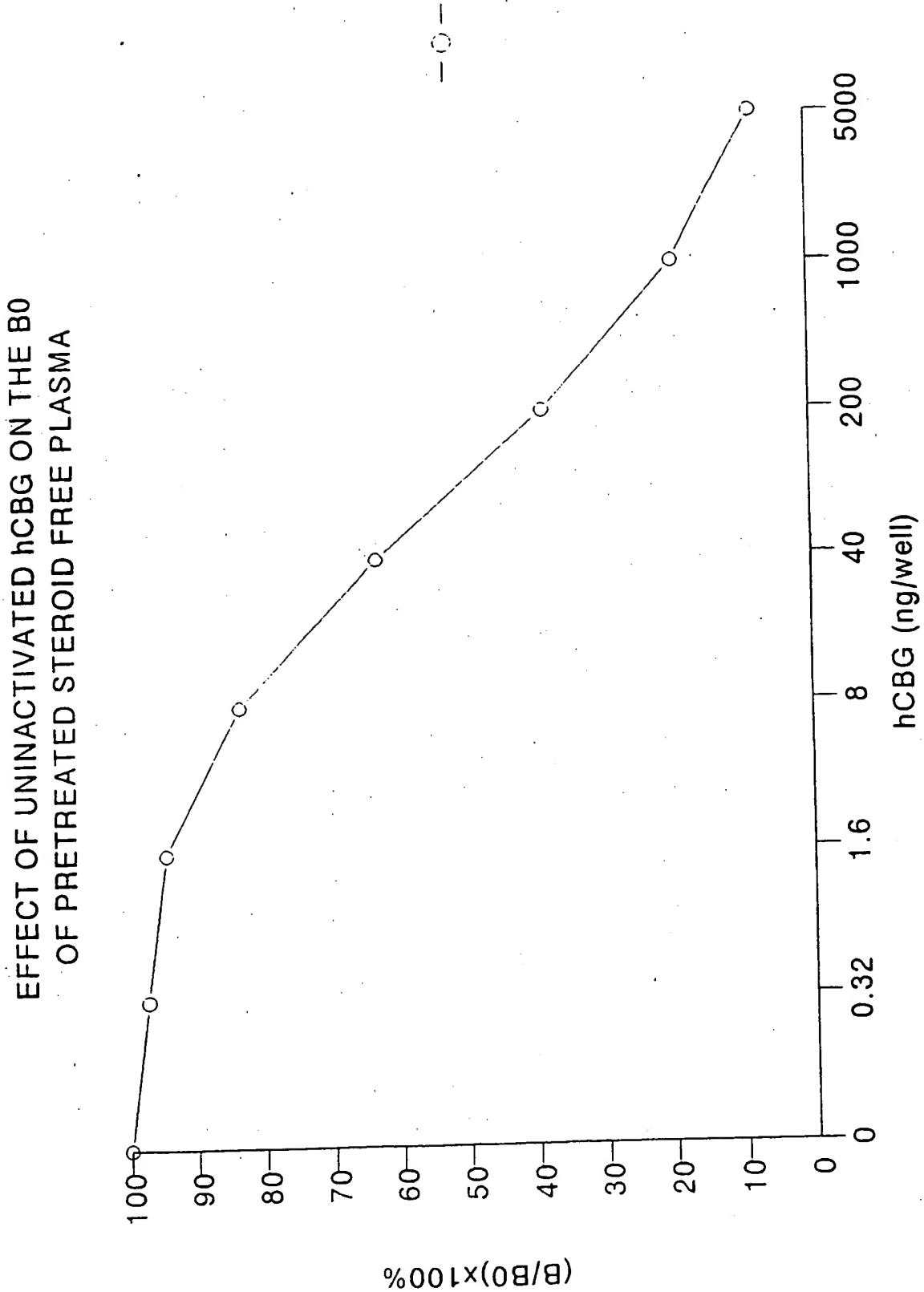
EFFECT OF UNINACTIVATED hCBG ON THE B0
OF PRETREATED STEROID FREE PLASMA
(B/B0)x100%
100
90
80
70
60
50
40
30
20
10
0
0
0.32
1.6
8
40
200
1000
5000
hCBG (ng/well)

Fig. 5.

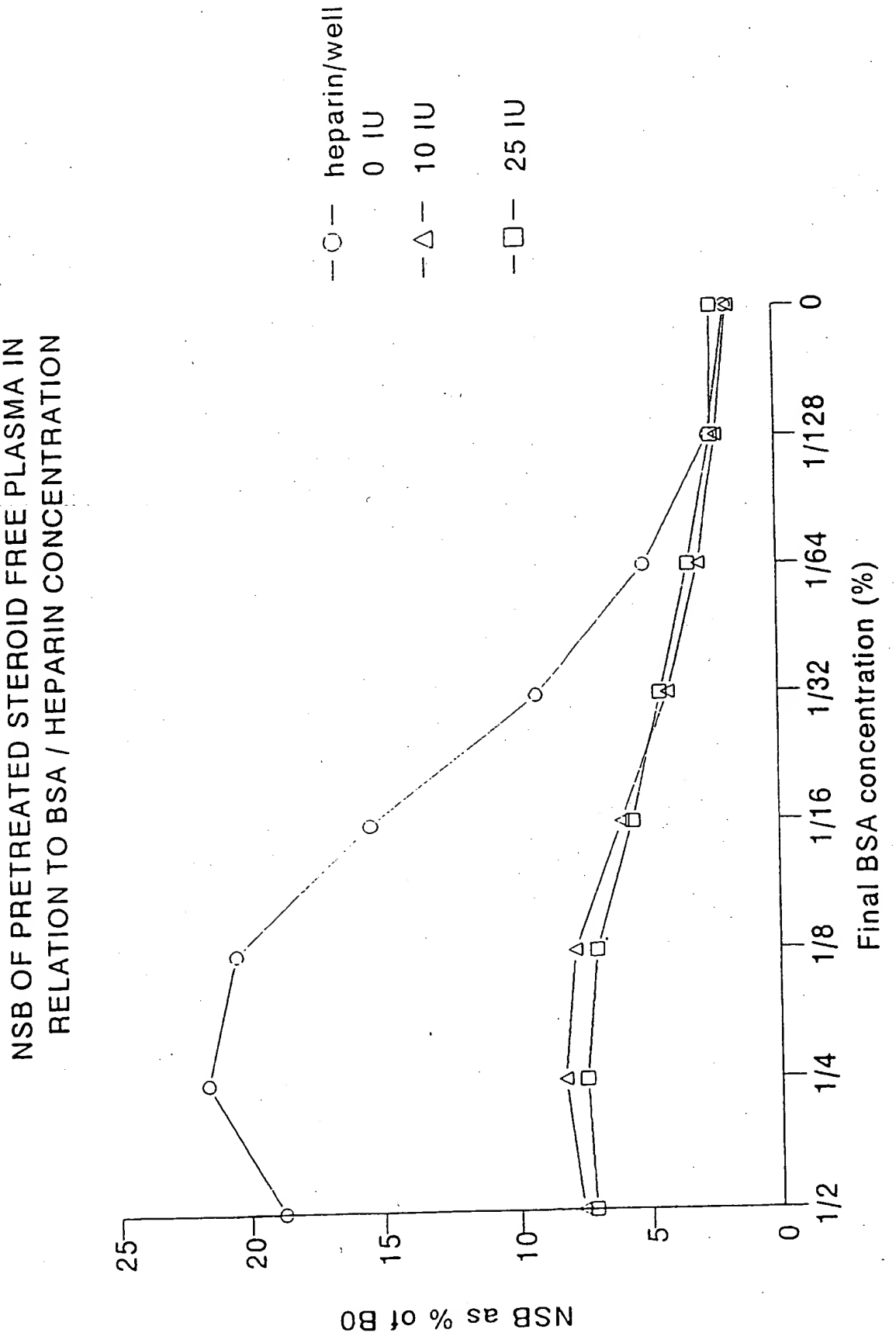
NSB OF PRETREATED STEROID FREE PLASMA IN
RELATION TO BSA / HEPARIN CONCENTRATION
heparin/well
0 IU
10 IU
25 IU
NSB as % of B0
25
20
15
10
5
0
1/2
1/4
1/8
1/16
1/32
1/64
1/128
0
Final BSA concentration (%)

Fig. 6.

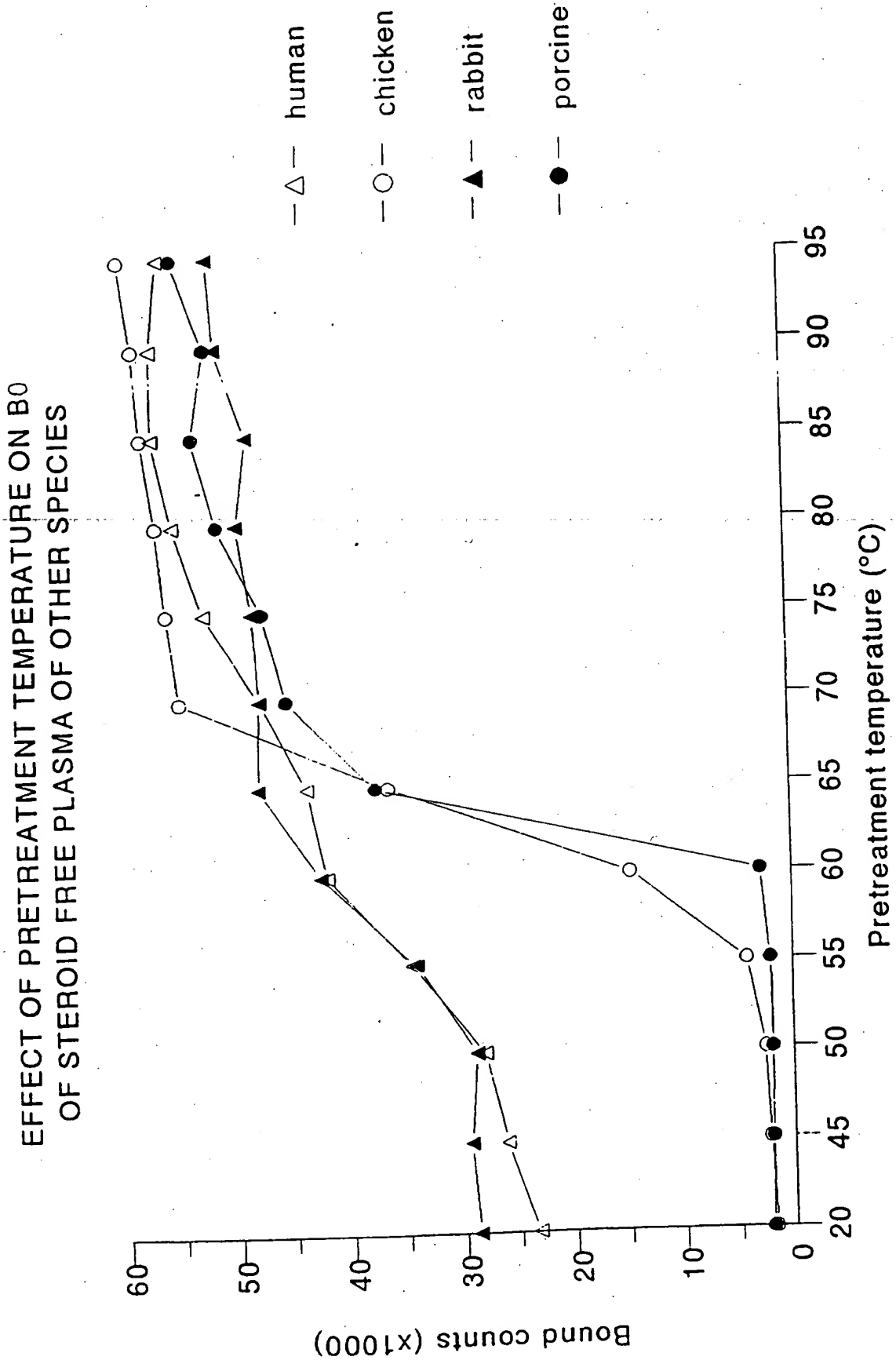
EFFECT OF PRETREATMENT TEMPERATURE ON B0
OF STEROID FREE PLASMA OF OTHER SPECIES
human
chicken
rabbit
porcine
Bound counts (x1000)
0
10
20
30
40
50
60
Pretreatment temperature (°C)
20
45
50
55
60
65
70
75
80
85
90
95

Fig. 7

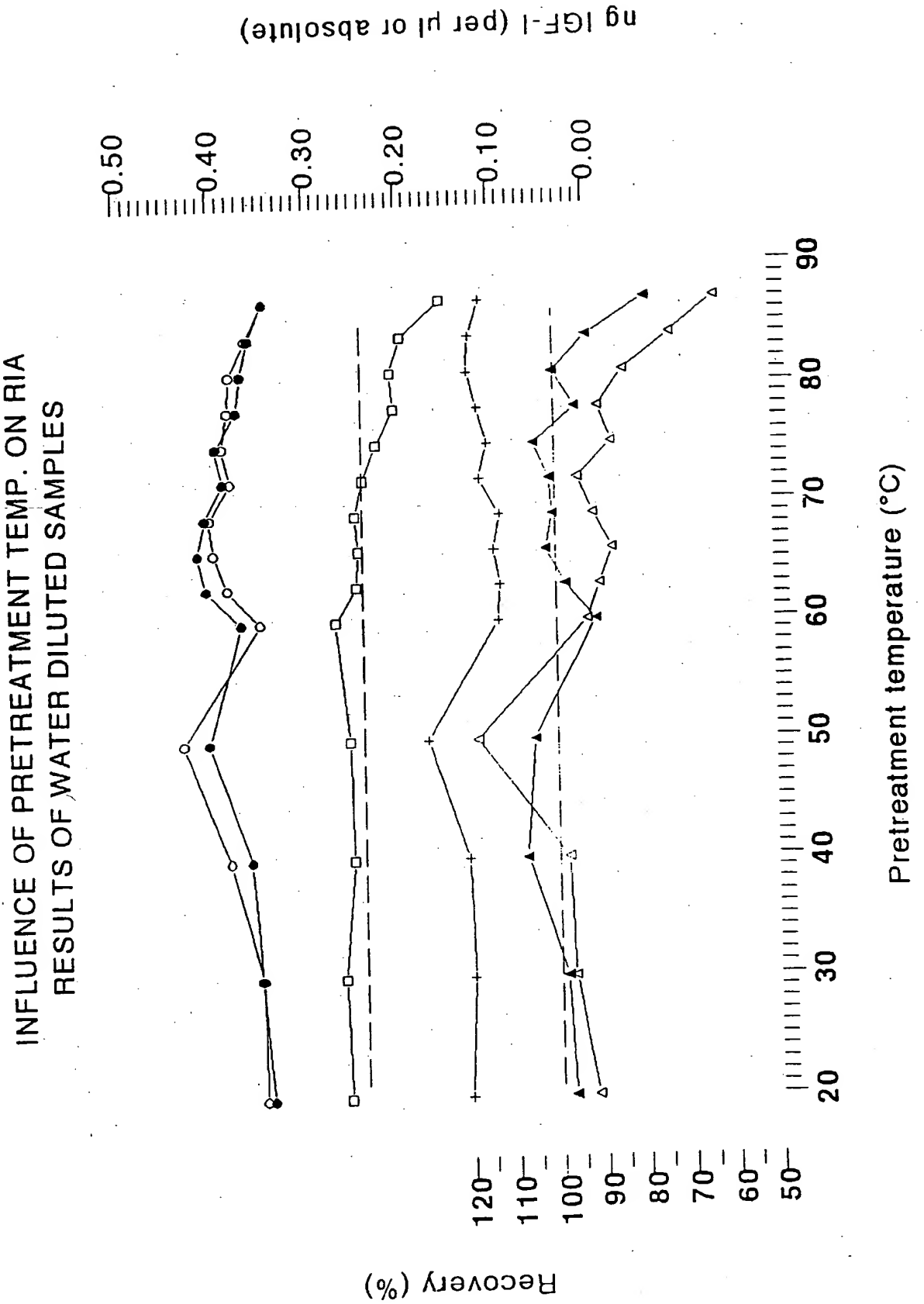
INFLUENCE OF PRETREATMENT TEMP. ON RIA
RESULTS OF WATER DILUTED SAMPLES
ng IGF-I (per µl or absolute)
0.50
0.40
0.30
0.20
0.10
0.00
Recovery (%)
120
110
100
90
80
70
60
50
20
30
40
50
60
70
80
90
Pretreatment temperature (°C)

Fig. 8

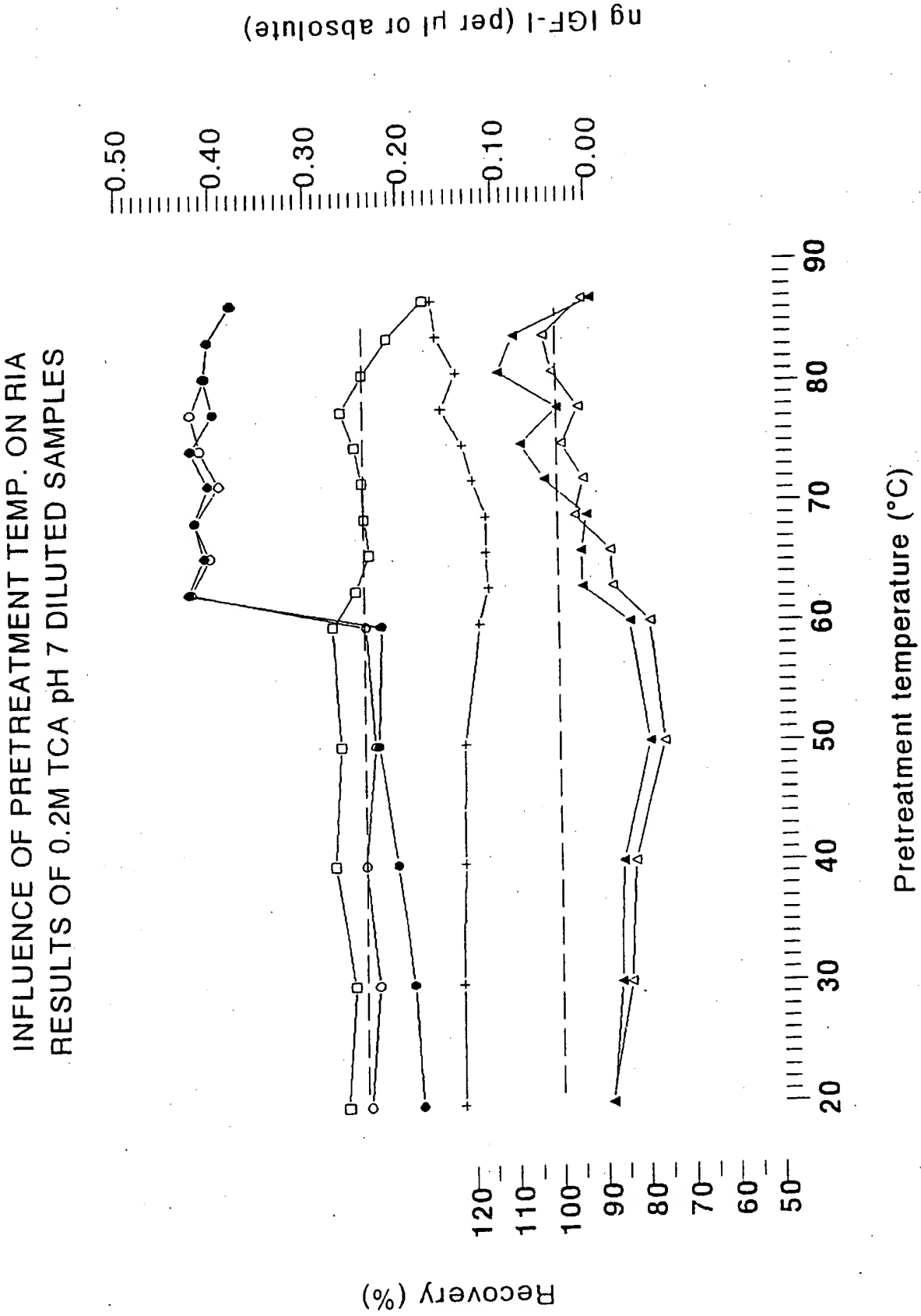
INFLUENCE OF PRETREATMENT TEMP. ON RIA
RESULTS OF 0.2M TCA pH 7 DILUTED SAMPLES
ng IGF-I (per µl or absolute)
0.50
0.40
0.30
0.20
0.10
0.00
Recovery (%)
120
110
100
90
80
70
60
50
20
30
40
50
60
70
80
90
Pretreatment temperature (°C)

Fig. 9

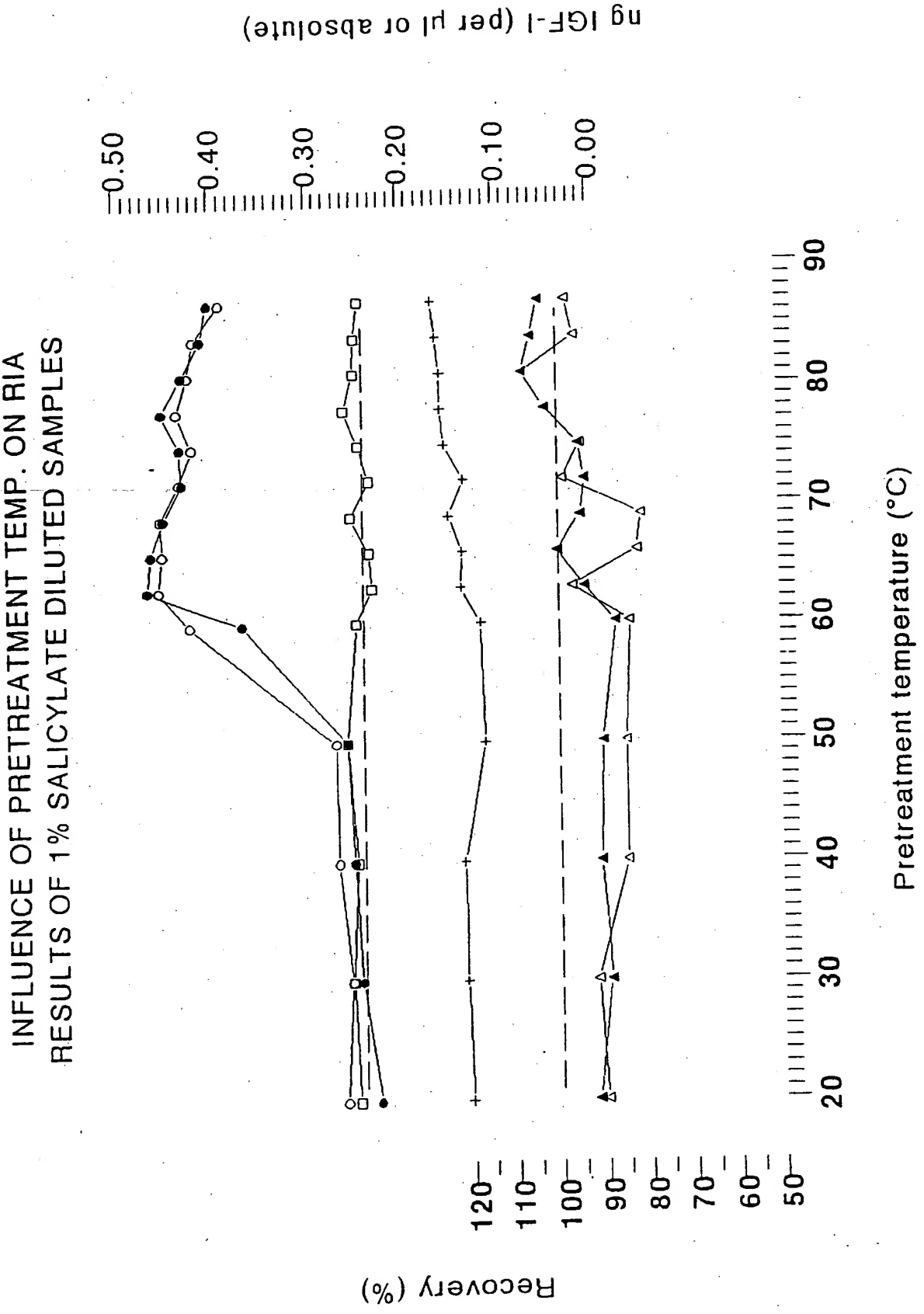
INFLUENCE OF PRETREATMENT TEMP. ON RIA
RESULTS OF 1% SALICYLATE DILUTED SAMPLES
ng IGF-1 (per µl or absolute)
0.50
0.40
0.30
0.20
0.10
0.00
Recovery (%)
120
110
100
90
80
70
60
50
Pretreatment temperature (°C)
20
30
40
50
60
70
80
90

Fig. 10

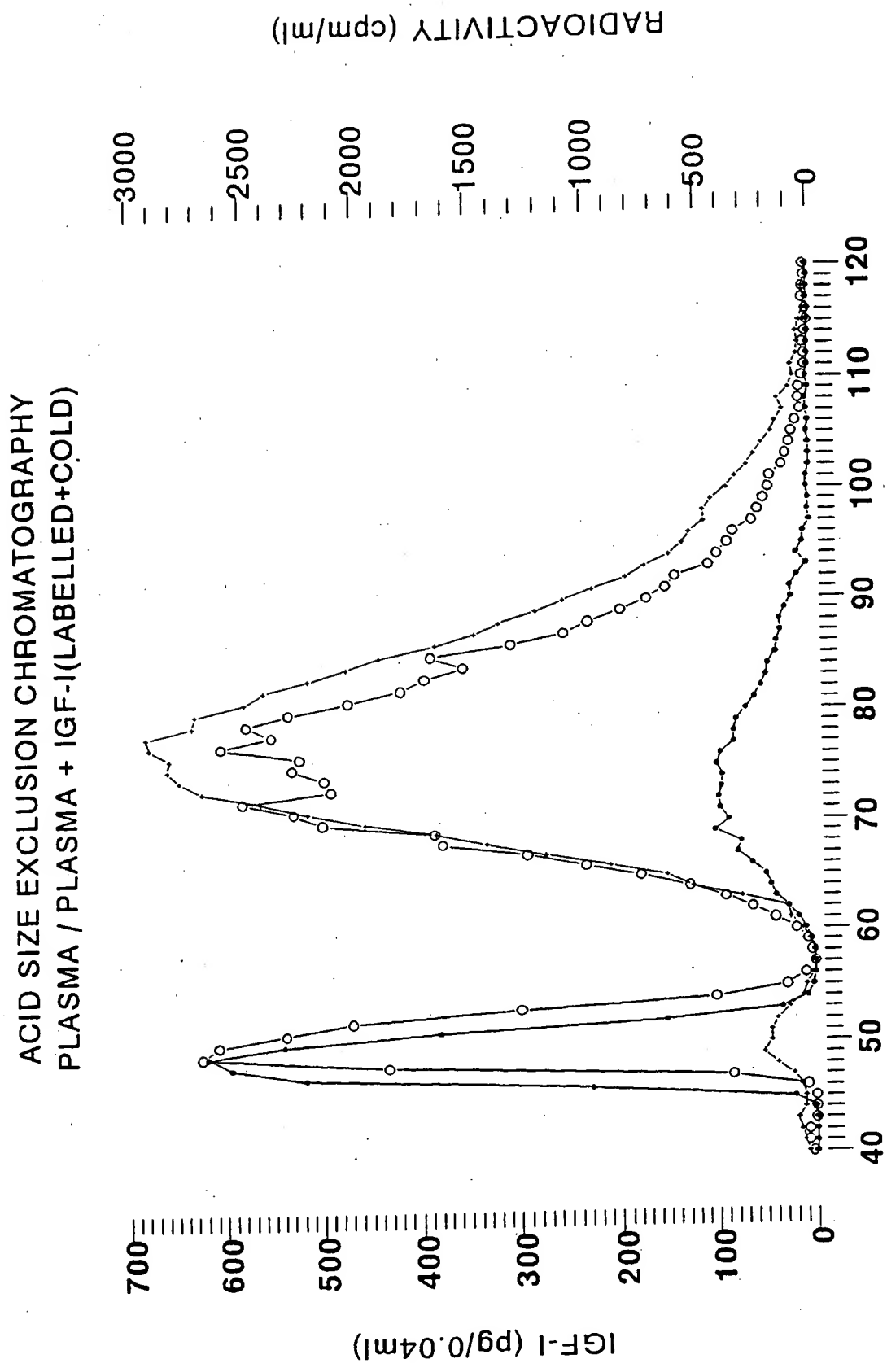
ACID SIZE EXCLUSION CHROMATOGRAPHY
PLASMA / PLASMA + IGF-I(LABELLED+COLD)
IGF-I (pg/0.04ml)
RADIOACTIVITY (cpm/ml)
FRACTION NUMBER
0
100
200
300
400
500
600
700
0
500
1000
1500
2000
2500
3000
40
50
60
70
80
90
100
110
120

Fig. 11

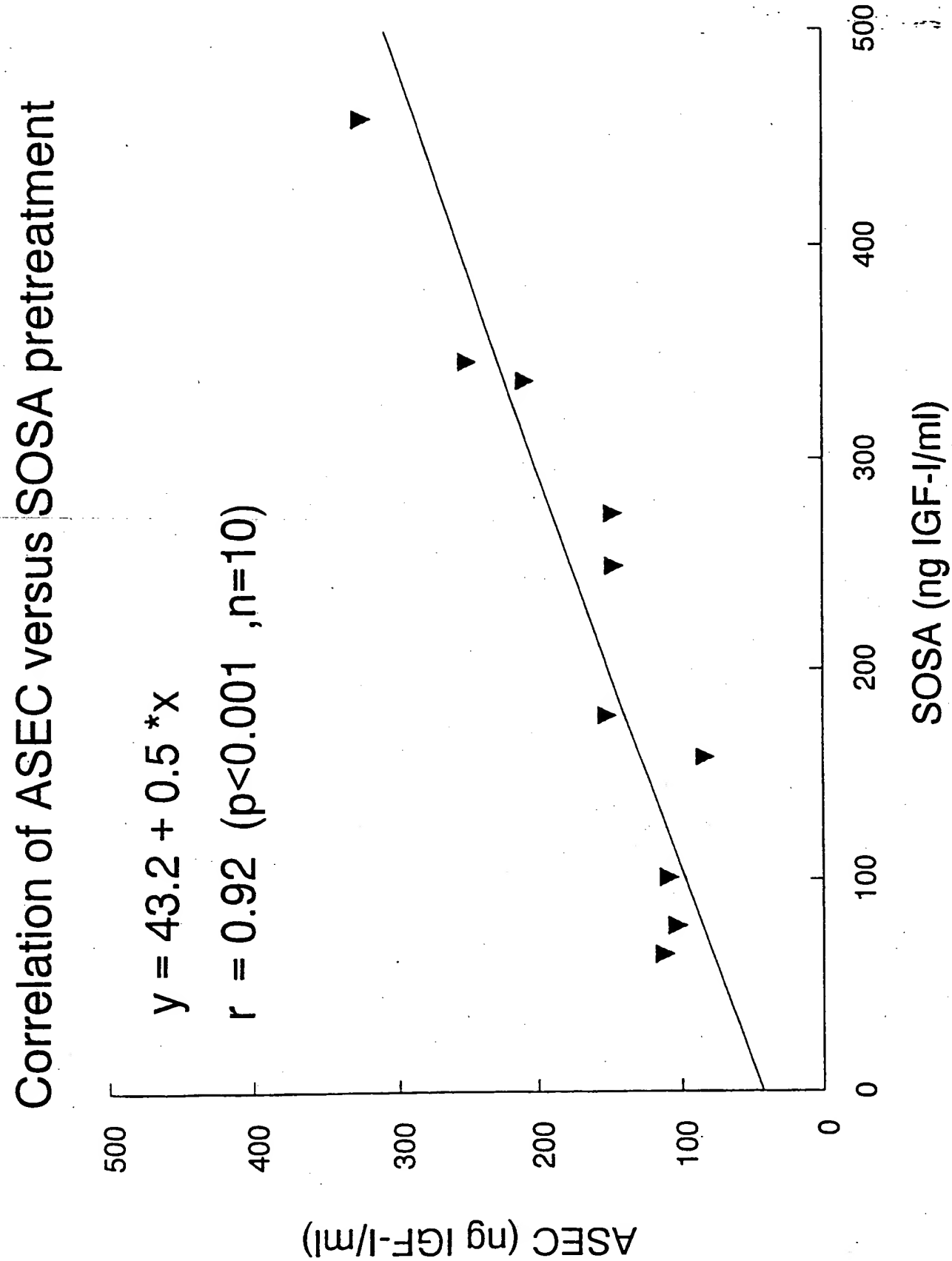
Correlation of ASEC versus SOSA pretreatment
y = 43.2 + 0.5 *x
r = 0.92 (p<0.001 ,n=10)
ASEC (ng IGF-I/ml)
SOSA (ng IGF-I/ml)
0
100
200
300
400
500

Fig. 12

INTERNATIONAL SEARCH REPORT

Inte. nal Application No
PCT/NL 98/00718

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/74 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 18, no. 373 (P-1769), 13 July 1994 & JP 06 102275 A (TOSOH CORPORATION) see abstract	1-6
Y	--- -/--	7-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 1999	24/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/NL 98/00718

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 99, no. 15, 10 October 1983 Columbus, Ohio, US; abstract no. 116263, XP002092750 see abstract & B. BUDZISZEWSKA ET AL.: "Radioimmunoassay method of androgen (testosterone and dihydrotestosterone) determination in human male blood serum without extraction and chromatography." ENDOCRYNOL. POL., vol. 34, no. 1, 1983, pages 61-70, Krakow Pol. ---	7-21
X	CHEMICAL ABSTRACTS, vol. 122, no. 5, 30 January 1995 Columbus, Ohio, US; abstract no. 47066, XP002065495 see abstract & Y. IKEGAMI: "Studies on the hormone-binding function of thyroid hormone-binding proteins by heat treatment" NIPPON NAIBUNPI GAKAI ZASSHI, vol. 70, no. 9, 1994, pages 017-1028, OSAKA JP ---	1
A	US 4 332 786 A (M.D. CABELLI ET AL.) 1 June 1982 see the whole document -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/NL 98/00718

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4332786 A	01-06-1982	NONE	